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CENTRAL NERVOUS FUNCTION AND CHANGES IN BRAIN METABOLITE CONCENTRATION: CHARACTERISTIC GLYCOGEN INCREMENT PATTERNS PRODUCED BY CONVULSANT DRUGS

BY

M. R. A. CHANCE

From the Pharmacology Department, University of Birmingham

(Received June 8, 1950)

In a previous publication (Chance and Yaxley, 1950), it has been reported that a rise in brain glycogen occurred during convulsions in mice. The brain glycogen rose only when the precipitating stimulus was of sufficient strength to produce a convulsion. In this sense the glycogen increment was found to be specifically associated with convulsions. The amount of the increment was not only related to the intensity of extrinsic stimuli in normal mice at convulsive levels and above, but also was directly related to the severity of the convulsions. Moreover, this increment varied in the various different major subdivisions of the brain after a standard stimulus. It was therefore concluded that the glycogen increment in any part of the central nervous system could be taken as an indication of the amount of " nervous activity " arising from a particular region during convulsions. Seizures in man and animals show a variety of forms apart from a major change from a clonic to a tonic state with increasing severity. Similar differences exist between the patterns of convulsive movement produced by maximal doses of different central nervous stimulants and of other drugs producing convulsions. It was therefore thought worth investigating whether the patterns of glycogen increment would differ accordingly and be a reliable guide to the patterns of central nervous activity underlying the differences between the seizures. Thus the glycogen increment patterns produced by convulsive doses of a variety of substances, which are already well known for their action on the central nervous system at subconvulsive dose levels, have been plotted.

METHOD

Male mice of a single strain weighing 20–25 g. were used throughout. Individual animals were given the dose required to produce convulsions in 50 per cent of animals (CD50) in 10 ml./kg. body weight, by intravenous injection; they were killed at various and, whenever possible, comparable intervals of time after the injection, during the convulsion and sometimes up to ninety minutes after the convulsions. This procedure produced clonic convulsions in all those animals which convulsed. In this way the rate of rise of glycogen, which always reached its peak at the end of the convulsion, was assessed in different parts of the brain as the convulsion proceeded.

Drugs were dissolved in aqueous solution. The CD50 values, which had been previously determined on this strain of mice, were as follows: *dl*-amphetamine sulphate, 40 mg./kg.; sodium diphenyl-hydantoin, 60 mg./kg.; caffeine (alkaloid), 35 mg./kg.; nikethamide (*B.P.*), 50 mg./kg.; dibenamine hydrochloride, 50 mg. kg.; strychnine hydrochloride, 6.0 mg./kg.; picrotoxin (*B.P.*), 2.5 mg./kg.; insulin, 80 units/kg.; leptazol, 50 mg./kg.

The procedure for the extraction and estimation of glycogen and the separation of the brain into the different regions has been described elsewhere (Chance and Yaxley, 1950).

Results

Normal values for glycogen in the different regions of the brain are given in mg./100 g. in Table I.

TABLE I

GLYCOGEN CONTENT OF NORMAL MOUSE BRAIN

Part of brain	Glycogen mg./100 g.	S.D.	No. of samples
Cortex	7.5	±0.38	22
Mid-brain	27.5	±0.49	22
Medulla	17.4	±0.56	22
Spinal cord	23.5	±0.48	4
Cerebellum: Central lobe	27.2	±0.77	4
Cerebellum: Lateral lobes	24.1	±1.40	4

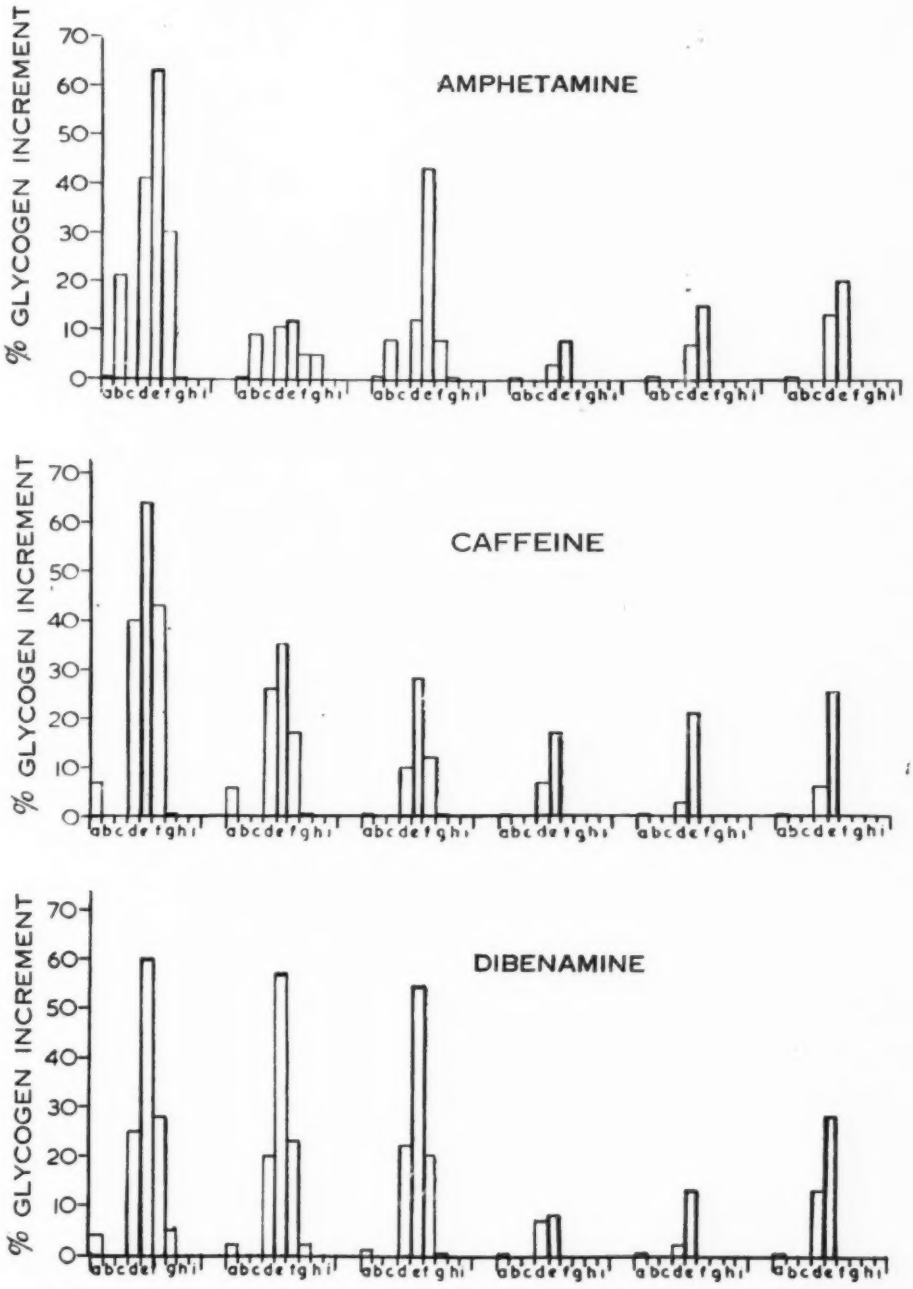
The results have been depicted in histograms (pp. 3–5) in which glycogen increments are expressed as percentages of the normal values for each part of the brain and are drawn against time for each drug. Each block in the histogram is the mean of three separate determinations. It has already been shown (Chance and Yaxley, 1950) that the variance of the increment is very low, and this has been found consistently throughout the present investigations. The mean standard deviation of the percentage increment, calculated from eighty sets of three determinations selected at random, was 1.65. The mean standard deviation, therefore, for the difference between two sets is $1.65\sqrt{2}$, which equals 2.33. Thus for a P value of 0.05 the difference between two percentage increments, each the result of three estimations, will be significant if they differ by more than 4.66 per cent ($2 \times 1.65\sqrt{2}$). This statement holds true for all levels of response, since the variance is independent of the response. All comparisons of the values for the individual blocks in the histograms are made on the assumption, therefore, that a difference has been recorded only if the means differ by 5 per cent or more. This criterion is slightly more rigorous than the statistical data warrant, but serves to emphasize the accuracy of the method.

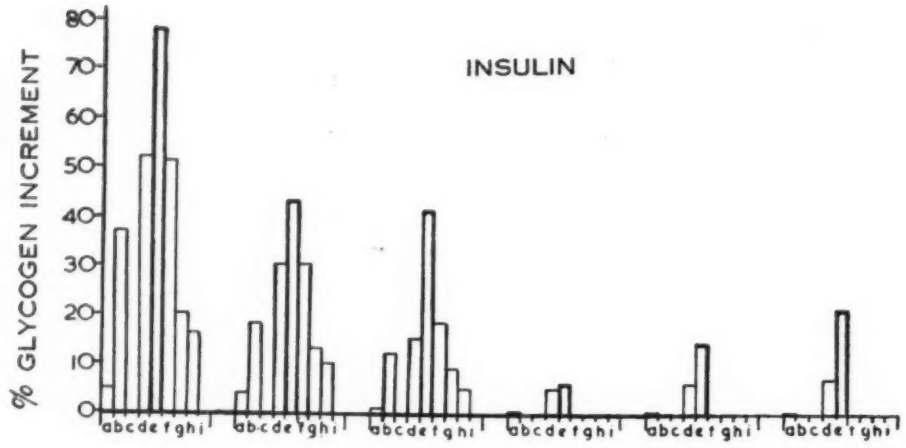
If these histograms are compared taking into account the rate of the rise in any one part of the brain (comparison eight seconds after start of convulsion), the peak rise and the relative rate of loss following the peak rise, it will be seen that each drug presents a distinctive pattern.

All the drugs except dibenamine and picrotoxin produced larger increments in the cortex than in other parts of the brain.

All the drugs produced smaller increments in the cerebellum and the spinal cord than in the other three parts of the central nervous system. Whereas all the other drugs produced a mid-brain increment greater than 34 per cent, amphetamine produced an increment in this region of only 13 per cent, which was about the same as that produced in the spinal cord and cerebellum. This is in marked contrast to the cortical and medullary increments, which were at least three times as large as that in the mid-brain.

HISTOGRAMS.—Ordinates: percentage glycogen increment. Abscissae: parts of the brain are arranged *from left to right* as follows: cortex, mid-brain, medulla, spinal cord, central lobes, and lateral lobe of the cerebellum. Time intervals are indicated along the abscissae by the letters *a* to *i*, thus: (*a*) preconvulsion; (*b*) start of convulsion; (*c*) 5 sec. after *b* for picrotoxin only; (*d*) during convulsion; (*e*) end of convulsion; (*f*) 20 min., (*g*) 40 min., (*h*) 60 min., and (*i*) 90 min. after convulsion.

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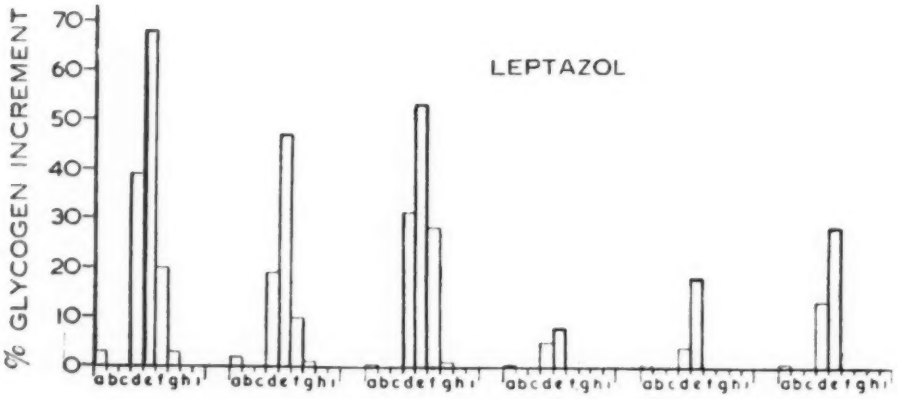
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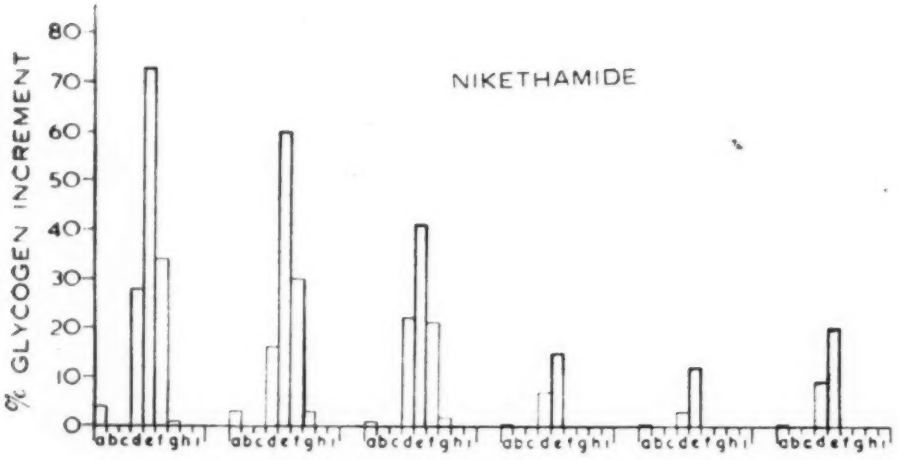
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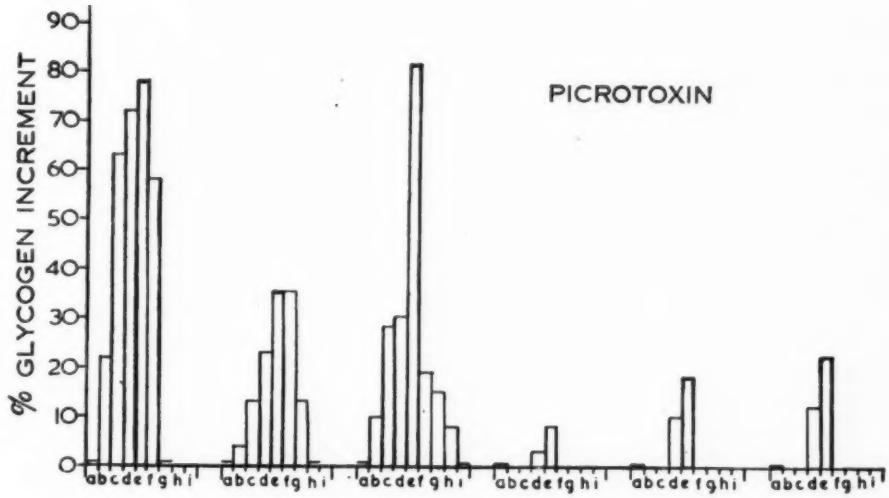
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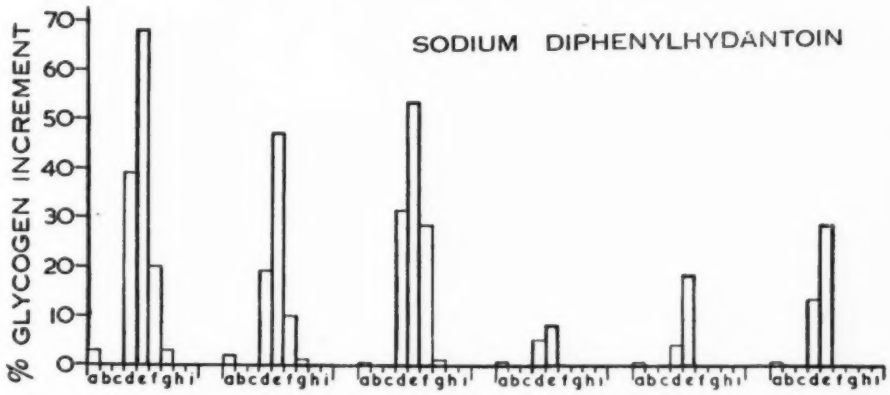
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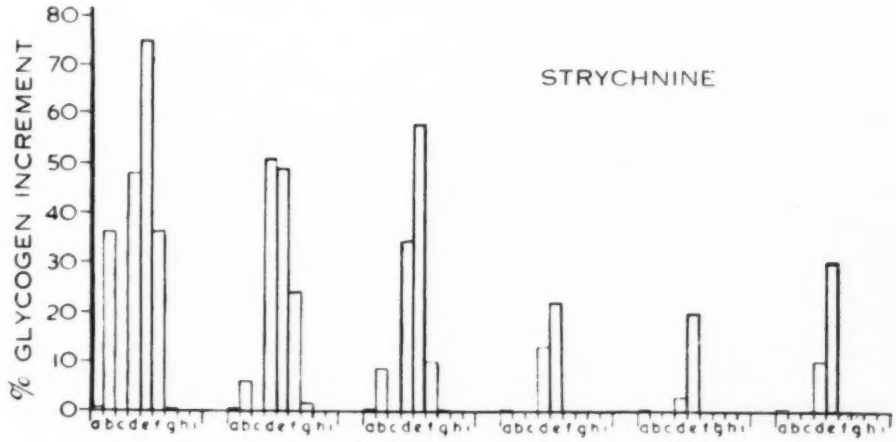
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The time elapsing between the injection of the drug and the onset of the convulsion was for most of the drugs no longer than thirty seconds, and the convulsion itself did not last longer than a minute. Picrotoxin was the only exception. The induction period for this drug lasted from two to four minutes and convulsions occurred sporadically over a period of a further five minutes. These facts must be remembered when assessing the relative rates of increment induced by the different drugs in a particular part of the brain. It will be noted that the apparently sudden increment in the medulla towards the end of the picrotoxin convulsions does not represent an exceptionally rapid rise whether this is estimated on the basis of the total time occupied by the convulsions or in relation to the time span itself.

If a comparison of the increments in the separate parts of the brain due to the different drugs is made (eight seconds after the start of the convulsion), it will be seen that four drugs (insulin, picrotoxin, amphetamine, and caffeine) produced a more rapid rise in the cortex than in the rest of the brain.

Picrotoxin stands out in contrast to all the others, in that it produced a final increment of glycogen in the medulla equal to, if not in excess of, the rise in the cortex, both these parts showing an 80 per cent increment. This cortical increment was only equalled during convulsions produced by insulin and by leptazol. Picrotoxin and amphetamine and to a lesser extent leptazol and sodium diphenyl-hydantoin produced larger increments in the medulla than they did in the mid-brain. Dibenamine was unique in that the increments in all the three higher regions of the brain were identical.

The rate of recovery was the same in the three higher parts of the brain after convulsions produced by caffeine, nikethamide, dibenamine, sodium diphenyl-hydantoin, and strychnine. The rate of recovery in the mid-brain after amphetamine convulsions was very slow, and this was probably due to the small extent of the rise which occurred in this part. Recovery was also noticeably slower in the later stages both in the mid-brain and medulla after picrotoxin convulsions and in the three higher regions after insulin convulsions.

Discussion

The evidence we have presented elsewhere (Chance and Yaxley, 1950), showing the intimate relationship of the glycogen increments with convulsive activity, suggested the present study, which describes the patterns of nervous activity characteristic of each drug. While at present there is not sufficient evidence to draw any conclusions about the relationship between convulsive activity in the central nervous system and the nervous activity of the cells during the convulsion, it is probable that there is some direct relation between these two phenomena. The method has demonstrated that characteristic patterns of glycogen increment are produced by a number of central nervous convulsions, and it is reasonable to infer that this reflects a patterned interference with the biochemical processes involved in the early stages of carbohydrate utilization. It is, however, not possible to infer that the differential increments are a result of the direct action of the drug on the particular part of the brain in which the increment has occurred. Before this is known it will be necessary to assess how far the activity engendered in one part of the brain modifies the increment in another.

The increments are expressed as a percentage of the normal values of the particular part of the brain under examination. The absolute values for each part differ considerably, and this (on the assumption that the glycogen is intracellular) must depend upon the number of neurones present in a given sample, i.e., on the proportion of grey to white matter and glial tissue. Since the same percentage increment can be produced in all parts of the brain by electrical convulsions (Chance and Yaxley, 1950) it is logical to assume that there are no major inherent differences limiting the extent of glycogen increment in any one part. If this is so then we have shown that the amount of activity in the spinal cord and cerebellum during clonic convulsions produced by these drugs is much less than the activity in the higher brain centres.

Owing to the restricted use of picrotoxin as an antidote to barbiturate poisoning, our knowledge of this drug, unlike that of the majority of central nervous stimulants, is derived from the use of doses which in the absence of the antidote would induce convulsions. It is interesting to compare, therefore, the action of this drug as revealed by the " convulsive " pattern with what we know about it. Picrotoxin produces, as might be expected, its maximum increment in the medulla, which is consistent with our knowledge that it is a potent stimulant of the respiratory centre. But it is also equally effective in stimulating the cortex; in fact the stimulation appears to start in the cortex, and to last longer there than in the medulla. It is interesting to note that, as early as 1875, Crichton Browne suggested that picrotoxin was " more than a medullary stimulant " and had some action on the cerebral cortex. It may be the reason for its peculiar effectiveness as an antidote to barbiturates, and in particular may be the explanation why the barbiturates can be " titrated " against it and *vice versa*. On the basis of the similarity between the glycogen increment curve of picrotoxin and leptazol we should expect that the latter substance would be the next most effective antidote, and indeed it is. Strychnine, which has some slight antidotal properties against lethal doses of barbiturates, showed some similarity to the aforementioned drugs, in that the increment in the medulla is of the same order as that produced by leptazol. It differed, however, from them in that the increment in the mid-brain comes very close to that in the medulla. It is interesting to note that this substance, regarded as a spinal stimulant, produced the largest spinal increment (23 per cent) of all the drugs examined.

Summary

The very low variance associated with the estimation of glycogen in mouse brain during procedures which bring about changes in amount of this metabolite makes it possible to estimate the relative changes in different parts of the brain with exceptional accuracy. The accumulation of glycogen in the brain of the mouse, which takes place during a convulsion, has been estimated in different parts of the central nervous system during seizures produced by convulsant drugs. The resulting patterns are interpreted as indicating the *distribution* of nervous activity in the brain arising from the stimulation of the drug.

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Brit. J. Pharmacol. (1951), 6, 8.

THE QUANTITATIVE SEPARATION OF ADRENALINE AND *NOR*ADRENALINE IN BIOLOGICAL FLUIDS AND TISSUE EXTRACTS

BY

T. B. B. CRAWFORD AND A. S. OUTSCHOORN*

From the Department of Pharmacology, University of Edinburgh

(Received October 11, 1950)

The qualitative similarity in the biological effects and chemical reactions of adrenaline and *nor*adrenaline has made difficult the determination of these substances when present together in biological fluids or tissue extracts. Several methods, however, have been devised. For the purpose of review these methods may be conveniently divided into two groups, pharmacological and chemical.

The pharmacological methods are based on the quantitative difference in the activities of the two amines on certain pharmacological preparations. In a number of such procedures parallel quantitative assays are carried out on at least two preparations: one of these is much more sensitive to adrenaline than to *nor*adrenaline, e.g., the isolated uterus of the non-pregnant rat or the isolated rectal caecum of the hen or chick; whereas the other is more sensitive to *nor*adrenaline or has the same sensitivity to both amines, e.g., the isolated colon of the rat, the perfused ear of the rabbit, the cat's blood pressure, or the denervated nictitating membrane of the cat (Bergström, Euler, and Hamberg, 1950; Bülbring, 1949; Gaddum and Lembeck, 1949; Gaddum, Peart, and Vogt, 1949; Mann and West, 1950). By the use of mathematical formulae incorporating the results of the assays in terms of one or other of the two amines and the activity ratio of the two amines on each preparation the amount of adrenaline and of *nor*adrenaline in the mixture may be determined.

It will be evident that the estimates of adrenaline and *nor*adrenaline are interdependent. Any inaccuracy in the assays or in the comparison of the activities of the amines will be reflected in the values for both adrenaline and *nor*adrenaline. In addition the chance of inaccuracy might be expected to increase when biological fluids or tissue extracts are analysed, since other substances, present in unknown concentrations, may affect the assays to a degree which is not always assessable. Gaddum and Lembeck (1949) have shown by statistical calculations that such tests do provide convincing evidence that a mixture of active substances is present but that they do not give a reliable estimate of their concentration.

A method described by Burn, Hutcheon, and Parker (1950), which is a modification of that devised by Bülbring and Burn (1949), appears to be less open to such criticism. In this procedure contractions of a cat's normal nictitating membrane and the rise of blood pressure are recorded. The effects of unknown solutions are compared with those of various equipressor mixtures of the two amines and the percentage methylation calculated from the effect on the nictitating membrane. It is unfortunate that this method is of use only when there is available at least 10 μg. of the combined amines. With this quantity present in 1 ml. of solution, only one estimate of the proportion of adrenaline to *nor*adrenaline can be obtained. For accuracy the mean of several estimates has to be determined.

* Ceylon Government scholar.

Of the chemical methods which have been described, two parallel in principle the pharmacological methods first discussed in that they depend on the difference in the degree of reactivity of the two amines under certain experimental conditions. Both are colorimetric and involve the use of mathematical formulae to translate extinction coefficients into terms of adrenaline and *nor*adrenaline. Euler and Hamberg (1949) measure the intensity of the red colours of adrenochrome and *nor*adrenochrome produced when a mixture of adrenaline and *nor*adrenaline is treated with iodine under carefully defined conditions. Schuler and Heinrich (1948, 1949a) based their method on the yellow colours produced by the catechols when coupled with a relatively complex diazonium salt, again under strictly defined conditions. The employment of these methods is limited, *inter alia*, by their lack of sensitivity, some 20–40 μg. of the combined amines apparently being the minimum required for the determinations.

Goldenberg, Faber, Alston, and Chargaff (1949) have devised a method based on the paper chromatographic separation of *nor*adrenaline and adrenaline, phenol being used as solvent (James, 1948). An estimate of the adrenaline and *nor*adrenaline content is made by planimetry and semi-logarithmic calculation (Fisher, Parsons, and Morrison, 1948) of the Prussian blue spots obtained in the adrenaline and *nor*adrenaline positions in the developed and dried chromatogram by spraying with potassium ferricyanide (James, 1948) followed by ferric sulphate. The limit of sensitivity is not less than 2 μg. for each amine, and, since this quantity must be applied to the paper in a volume of 0.01 ml., it follows that the concentration of the solution must be somewhat high.

From this review of existing techniques for the determination of adrenaline and *nor*adrenaline in mixtures it would appear that, for many pharmacological experiments in which at the most only a few micrograms of the amines are present, none is wholly suitable. The chemical methods lack sensitivity while the biological methods, which in the main are highly sensitive, tend to be deficient in accuracy. This deficiency in accuracy could be overcome to a large extent by first separating the two amines so that each might be assayed individually against a standard solution of the appropriate amine on a suitably sensitive pharmacological preparation.

The account which follows is a description of the adaptation of the paper chromatographic technique described by James (1948) for the qualitative separation of adrenaline and *nor*adrenaline to the quantitative determination of these amines when present together in biological fluids and tissue extracts. The method involves the separation of the two amines by paper chromatography, elution of the separated amines from the appropriate portions of the developed chromatogram, and finally the determination of the adrenaline and *nor*adrenaline contents of the eluates by assay on the rat's blood pressure preparation. The sensitivity of the method appears to be limited only by that of the assay procedure.

METHODS

Separation of adrenaline and noradrenaline *by paper chromatography*

Chromatographic technique.—All chromatograms are carried out by the capillary ascent method of Williams and Kirby (1948), water-saturated phenol being used as solvent. Phenol (500 g.) is distilled at atmospheric pressure from zinc dust (20 g.) and the distillate, while still warm, shaken with a small excess of distilled water in a separating funnel. Sulphur dioxide is bubbled through the mixture for 10 min. and the mixture allowed to stand overnight to permit complete separation of the phases. The lower yellow phenolic layer (500 ml.) is poured into a clean dry glass tank (15 cm. $\times$ 32 cm. $\times$ 45 cm.) to give a layer

of fluid about 1.5 cm. in depth. The tank is closed by a glass plate carrying an inlet and an outlet tube at diagonally opposite corners, so that the air inside the tank can be replaced by another gas. The union of the top of the tank to the glass plate is made airtight by the liberal use of soft paraffin. The solvent may be used repeatedly, but it has been our practice to renew it once a week.

Preparation of the filter paper.—A sheet of Whatman No. 1 filter paper is cut and ruled off in pencil in the manner illustrated in Fig. 1. Two samples, each containing adrenaline and *nor*adrenaline, can be chromatographed on the one sheet of paper. The paper is

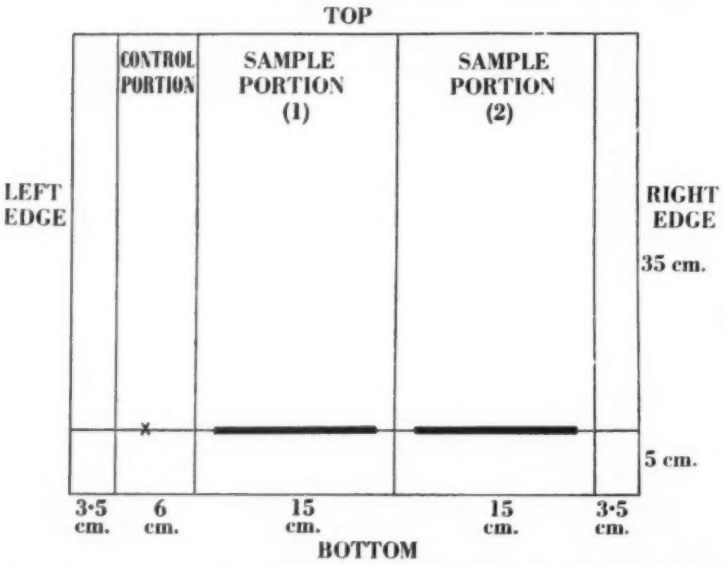

FIG. 1.—Diagram of method of ruling of sheet of filter paper for the chromatographic separation of adrenaline and *nor*adrenaline.

fashioned into cylindrical form by joining the left and right sides edge to edge by cellulose tape (1.6 cm. wide) applied along the length of the join to within about 2 cm. from the bottom of the paper. The edges of the paper at the join must not overlap, as any overlapping results in an uneven solvent flow extending some distance on either side of the join. The sample portions of the paper *only* are damped with a freshly prepared solution of ascorbic acid (50 mg. per 100 ml.). An all-glass spray should be used for this purpose. The paper is allowed to dry at room temperature, about 1.5–2 hr. being required.

Application of the test solution to the ascorbic acid treated paper cylinder.—The material to be analysed—usually in the form of a solution in ethanol acidified with concentrated hydrochloric acid (0.1 ml./100 ml. ethanol)—is applied along the "15 cm." line at the foot of the paper cylinder (thickened line in Fig. 1), care being taken that the fluid does not creep closer than 1.5 cm. to the boundary lines of the " sample portion " of the paper. The solution is best applied from a capillary pipette as a series of drops each approximately 0.005 ml. at a distance apart such that the drops just merge into one another when they spread on the paper. The width of the moisture band should not at any time exceed 1 cm. When the length of the line has been wetted, the alcohol is allowed to evaporate at room temperature and another series of drops applied. This process is continued until all the solution has been transferred to the paper. The whole process takes about an hour.

Aqueous solutions may also be treated in a similar manner, but in this case it is advantageous to apply the solution over the whole width of the two sample portions of the paper. This will balance to some extent the extra time required for the solvent to evaporate. Heat should not be applied to facilitate the evaporation of the solvent.

Finally, on to the " control portion " of the paper at a point 5 cm. from the foot of the cylinder and 2 cm. from the left-hand boundary line (point X in Fig. 1) is applied 0.01 ml. solution of adrenaline and *nor*adrenaline (10 mg. each amine/ml. 0.01 N–HCl).

Development of the chromatogram.—The test and control solutions having been applied, the paper cylinder is stood with its lower end immersed in the solvent in the glass tank. A tank of the dimensions already quoted will accommodate two such paper cylinders quite easily. The tank, standing in a well-ventilated fume cupboard, is closed with the glass plate and sulphur dioxide passed in for 15 min., after which the inlet and outlet tubes are closed. Development of the chromatogram is allowed to proceed for 20–24 hr., when the solvent will have risen some 25–30 cm. up the paper.

Removal of solvent from the developed chromatogram.—The paper cylinder is removed from the tank and the cellulose tape, both backing and adhesive, stripped off. The filter sheet is held at the dry end and pulled backwards and forwards three or four times through a layer of benzene (A.R.) contained in a large porcelain developing dish. Care is taken that the whole of the paper which has been wetted with phenol is washed with the benzene. The paper is then hung up to dry for about half an hour, after which time it may still feel slightly damp but will be sufficiently dry to handle.

Locating adrenaline and nor*adrenaline on the developed and dried chromatogram.*—The " control portion " of the paper is cut off and sprayed with a solution of 0.44 g. $K_3Fe(CN)_6$ in 100 ml. 0.2 M–phosphate buffer *p*H 7.8 (James, 1948). Care must be taken that none of this solution reaches the " sample portions " of the paper. *Nor*adrenaline and adrenaline form red oxidation products and show up on the paper strip as red spots, the *nor*adrenaline spot being the one nearer to the starting line of the chromatogram. The distances from the starting line to the lower and upper extremities of the two spots are measured. These measurements serve to locate the amines on the " sample portions " of the paper. Allowance has to be made, however, for the fact that in the chromatography of biological extracts the flow of the amines may be influenced by other materials present in the extracts. It is advisable, therefore, that a preliminary experiment be carried out in which adrenaline and *nor*adrenaline (0.1 ml. control solution is suitable) is added to an extract and the extract chromatographed in parallel with a normal control. Spraying of both chromatograms with the $K_3Fe(CN)_6$ reagent will give information as to the effect of substances in the extract on the flow of the amines. With plasma and adrenal gland extracts we have found that there is a tendency for the amines to flow slightly further than they do in pure solution. Accordingly, as a routine measure, we have corrected for this difference in the position of the amines in the " sample " and " control portions " of the paper by subtracting 1 cm. from the measurement of the lower extremity and adding 2 cm. to that of the upper extremity of the *nor*adrenaline control spot and subtracting 1 cm. and adding 1 cm. to the lower and upper limits respectively of the adrenaline control spot. Transverse strips conforming to these corrected measurements of the location of the two amines are cut from the " sample portions " of the paper. The strips, usually 6–7 cm. wide, are suitably marked in pencil for future identification.

Elution of the amines from the paper strips.—The amine contained in each strip is eluted therefrom with a solution containing 0.4 g. $NaH_2PO_4.2H_2O$ (A.R.) and 1 mg. ascorbic acid per 100 ml. The method of elution is similar to that described by Dent (1947). Each paper strip is cut to a point at one end, care being taken not to remove any paper on which adrenaline or *nor*adrenaline is likely to be present. The other end is inserted to a depth of about 0.5 cm. between two microscope slides held together by a narrow elastic band. The extruding portion of the strip is bent sharply over the edge of the slides so that it will hang vertically when the far sides of the slides are immersed in the eluting fluid. The pointed end of the paper just intrudes into the mouth of a test-tube or a 15-ml. centrifuge

tube. The eluting fluid passes between the microscope slides, on to and down the paper, the eluate dropping into the collecting tube. To prevent undue evaporation of the solvent from the papers, the elutions are carried out in a fairly airtight chamber containing a beaker of water. The elutions are allowed to continue overnight.

While any suitable vessel may be used as a reservoir for the elution fluid we have found it convenient, when a number of elutions have to be carried out simultaneously, to use a battery of troughs prepared from celluloid tubing. An 8-cm. length of celluloid tubing, 1.5 cm. internal diameter, is sectioned along its length about 1 mm. from a diameter and the deeper section closed at each end by a strip of celluloid. If such a trough be filled with 7.5 ml. eluting fluid, the volume of eluate collected in an overnight run will be 3–4 ml. By thus restricting the supply of eluting fluid, volumes of eluates will be obtained which are fairly constant and the quantity of NaH_2PO_4 in the eluate will give rise eventually to an almost isotonic solution for the biological assay.

Preparation of eluates for biological assay.—To remove traces of phenol each eluate is shaken vigorously three times with equal volumes of benzene (A.R.), the benzene layers after separation being sucked off through a capillary pipette. The aqueous layer is transferred with washing with 1–2 ml. distilled water to a 50 ml. R.B. Quickfit and Quartz flask and evaporated *in vacuo* at 55–60° (external temperature). Heating is stopped when the fluid has evaporated almost but not completely to dryness. The flask and contents are allowed to cool slowly to room temperature with the suction still in operation. The residue in the flask is taken up in 1.00 ml. ascorbic acid solution (50 μg./ml.) and the adrenaline or *nor*adrenaline content of this solution determined by assay on the rat's blood pressure preparation. The solutions are kept below 5° until the assays can be performed.

Assay of adrenaline and nor*adrenaline by the rat's blood pressure preparation*

Preparation of the rat.—The rat's blood pressure preparation has been modified from that of Landgrebe, Macaulay, and Waring (1946).

A rat (200–250 g.) of either sex is anaesthetized with urethane (175 mg. or 0.7 ml. of a 25 g./100 ml. aqueous solution per 100 g. body weight subcutaneously) and then tied in a supine position to a board. Through a mid-line incision in the neck the trachea is cannulated and one carotid artery dissected ready for cannulation. The other carotid artery is tied off and loose ligatures placed round both vagi. A cannula is tied into a femoral vein and fixed firmly by a ligature embracing it and the thigh together. The distal end of the venous cannula is joined by a short piece of rubber tubing to a 1-ml. burette fitted, just above the tap, with a side arm which is connected by means of a piece of rubber tubing with a spring clip to a reservoir containing warm saline. The burette can thus be refilled without detaching it. Heparin (2 mg./100 g. rat) is injected through the venous cannula and washed in with saline. The arterial cannula is then tied in, fixed in a clip, and connected to a special mercury manometer, normal saline being used as the liquid bridge. Both vagi and the femoral nerve lateral to the venous cannula are cut. Before commencing the assays the preparation is left for 15–20 min. until the blood pressure settles to a steady level. Once set up the rat should not be touched, especially when injections are made. The slightest handling causes marked changes in the blood pressure of the animal.

All the cannulae used in this preparation are of the conventional pattern but of suitable size, with orifices of about 2.5 mm. external diameter for the tracheal cannula and 1 mm. external diameter for the arterial and venous cannulae, the internal diameters being slightly less. The special mercury manometer used is the subject of an addendum to this paper.

Preparation of standard solutions of adrenaline and nor*adrenaline.*—Standard solutions of adrenaline (2 μg./ml.) and *nor*adrenaline (1 μg./ml.) are made up from stock solutions by dilution in normal saline containing freshly dissolved ascorbic acid (50 μg./ml.). The

stock solutions of the amines, each containing 1 mg./ml. of the base, were prepared from synthetic (−) adrenaline (B.W. and Co.) and (−) *nor*adrenaline (+) bitartrate monohydrate (Bayer) by solution in 0.01 N–HCl. Such stock solutions appear to remain stable for several months if stored in small stoppered test-tubes at 5°.

Assay routine.—Test samples containing adrenaline are assayed against standard adrenaline and those containing *nor*adrenaline against standard *nor*adrenaline solutions. If required, dilution of a test solution is made in normal saline containing freshly dissolved ascorbic acid (50 μg./ml.). Test and standard solutions, both at room temperature, are injected alternately into the venous cannula of the rat preparation by means of a 1-ml. tuberculin syringe. The volume injected may be varied from 0.025 ml. to 0.4 ml., the dose being immediately washed in with a quantity of normal saline such that the total volume of fluid given at any one time is 0.6 ml. This total volume of fluid may be varied with the circumstances but should, of course, be the same for both test and standard in any one assay. In our hands the greatest accuracy is obtained when the total volume does not exceed 0.4 ml. Injections are made at regular intervals, usually every two minutes. It has been our custom in carrying out these assays to keep the dose of the test solution constant, and to bracket and finally match the responses with varying doses of the standard solution. An illustration of a record from a typical assay by the rat's blood pressure preparation is given in Fig. 2.

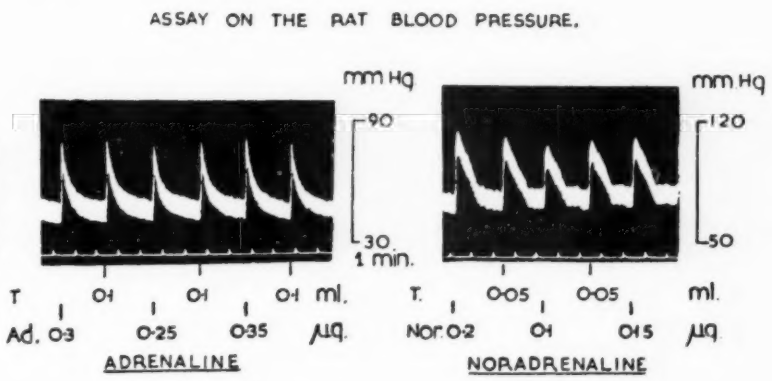

FIG. 2.—Illustrations of typical records obtained in the assay of adrenaline and *nor*adrenaline by the rat's blood pressure preparation.

RESULTS

Application of the method to the quantitative separation of adrenaline and nor*adrenaline in simple solution, biological fluids, and tissue extracts*

The results quoted below are representative of those obtained by the application of the method to the analysis of simple solutions and extracts containing adrenaline and *nor*adrenaline. The figures given in parentheses in the Tables are the upper and lower limits of the estimates of the assay, the smaller figure being definitely less than the true value of the unknown and the larger definitely greater as observed from the blood pressure responses.

Simple solution.—Mixtures of adrenaline and *nor*adrenaline in 1.00 ml. acid-ethanol (0.1 ml. conc. hydrochloric acid/100 ml. ethanol) were analysed. The recoveries are shown in Table I.

Plasma.—Blood was obtained from rabbits, after the injection of 5 mg. heparin, by bleeding from an ear vein into a beaker surrounded by ice. The plasma was

TABLE I

RECOVERY OF ADRENALINE AND *nor*ADRENALINE FROM MIXTURES IN SIMPLE SOLUTION

Mixture	Adrenaline			*Nor*adrenaline		
	Added μg.	Found μg.	Recovery %	Added μg.	Found μg.	Recovery %
	Series 1. Recovery experiments carried out at different times					
a	1.0	0.75 (0.5 – 1.0)	75	10.0	7.5 (5.0–10.0)	75
b	1.0	1.6 (1.33– 2.0)†	160	10.0	7.5 (5.0–10.0)	75
c	2.0	2.0 (1.33– 4.0)	100	Nil	<0.062*	—
d	2.0	2.0 (1.33– 4.0)	100	Nil	<0.062*	—
e	2.0	2.0 (1.5– 2.5)	100	Nil	<0.062*	—
f	2.0	1.5 (1.0–2.0)	75	Nil	<0.062*	—
g	10.0	8.0 (6.0–12.0)	80	1.0	0.75 (0.5–1.0)	75
h	10.0	15.0 (10.0–20.0)†	150	1.0	0.75 (0.5–1.0)	75
Series 2.	Recovery experiments done simultaneously. All assays carried out on the same rat preparation					
j	0.25	0.19 (0.13–0.25)	75	0.13	<0.25*	—
k	0.50	0.38 (0.25–0.5)	75	0.25	<0.25*	<100
l	1.0	1.0 (0.75–1.5)	100	0.50	0.31 (0.25–0.38)	62
m	2.0	2.0 (1.0–4.0)	100	1.0	0.75 (0.5–1.0)	75
n	2.0	2.0 (1.5–2.5)	100	0.10	<0.25*	—
p	2.0	2.0 (1.5–2.5)	100	0.25	<0.25*	100
q	2.0	2.5 (2.0–3.0)	125	0.50	0.25 (0.13–0.38)	50
r	20.0	15.0 (10.0–20.0)	75	1.0	0.75 (0.5–1.0)	75
s	Nil	<0.25*	—	Nil	<0.25*	—

* This figure represents the minimum quantity of amine/ml. solution which on injection of 0.4 ml. into the rat preparation would give a pressor response just noticeably different from that produced by the injection of an equal volume of saline. Thus concentrations below this figure cannot be assayed.

† Discrimination of rat preparation poor.

separated by centrifugation (3,000 r.p.m. for 10 min.) in a tube packed in ice. Adrenaline-*nor*adrenaline mixtures in plasma were made up by diluting 0.25 ml. of an appropriate standard solution of adrenaline and a like volume of *nor*adrenaline solution in 0.01 N–HCl with 2 ml. plasma. Of this mixture, 1 ml. was taken for analysis.

For the paper chromatography it was necessary that the protein be first removed from the plasma. This was effected by precipitation with acid-ethanol. To 10 ml. cold acid-ethanol (0.05 ml. conc. HCl/100 ml. ethanol) in a 15-ml. centrifuge tube, 1 ml. adrenaline-*nor*adrenaline-plasma mixture was added drop by drop. The tube was stoppered and the mixture thoroughly shaken and left at 5° for 1.5 hr. The underside of the stopper was washed into the main bulk of the fluid with 1 ml. acid-ethanol and the mixture centrifuged 15 min. at 4,000 r.p.m. The clear and colourless supernatant was transferred to a 50-ml. Quickfit and Quartz R.B. flask, the inside walls of the tube being washed down into the flask with 2 ml. acid-ethanol. The solution was evaporated to dryness *in vacuo* at 55–60° (external temperature), the flask being allowed to cool slowly to room temperature before releasing the suction. The faintly yellow lipid-like residue was leached with 0.75 ml. acid-ethanol (0.1 ml. conc. HCl/100 ml. ethanol) and the extract applied to a filter-paper cylinder. A further extract with 0.5 ml. acid-ethanol was also applied. Results obtained with this procedure are given in Table II.

TABLE II

RECOVERY OF ADRENALINE AND *nor*ADRENALINE FROM MIXTURE IN PLASMA

The results for individual members of each group of experiments were obtained from assays on the same rat preparation. 1 ml. plasma-adrenaline-*nor*adrenaline mixture was used for each analysis

Sample	Adrenaline			*Nor*adrenaline		
	Added μg.	Found μg.	Recovery %	Added μg.	Found μg.	Recovery %
1a	10.0	10.0 (6.7–12.3)	100	2.0	1.5 (1.0–2.0)	75
1b	2.0	2.0 (1.5–3.0)	100	10.0	7.5 (5.0–10.0)	75
1c	2.0	2.0 (1.5–3.0)	100	10.0	7.5 (5.0–10.0)	75
1d	Nil	<0.5*	—	Nil	<0.13*	—
2a	10.0	10.0 (7.5–15.0)	100	2.0	1.5 (1.0–2.0)	75
2b	10.0	10.0 (7.5–15.0)	100	2.0	1.5 (1.0–2.0)	75
2c	2.0	2.0 (1.5–3.0)	100	10.0	5.0 (3.3–6.7)	50
2d	2.0	2.0 (1.5–3.0)	100	10.0	7.7 (6.7–10.0)	77
2e	Nil	<0.25*	—	Nil	<0.13*	—
3a	10.0	10.0 (7.7–12.5)	100	1.0	1.0 (0.5–1.5)	100
3b	1.0	0.8 (0.67–1.0)	80	10.0	10.0 (5.0–15.0)	100
3c	Nil	<0.25*	—	Nil	<0.13*	—

* See footnote to Table I.

Adrenal glands.—As an example of the application of the method to tissue extracts, some results obtained in the analyses of adrenal glands from 15 normal rats are given in Table III. The standard deviations shown in this test are an indication of the variability of the rats and not of the error of the method.

TABLE III

DETERMINATION OF ADRENALINE AND *nor*ADRENALINE IN ADRENAL GLANDS OF NORMAL RATS

Mean estimates ± standard deviation from 15 rats (150–200 g.)

Amine	μg./100 g. body weight	μg./mg. gland	Percentage combined amines (mean value)
Adrenaline	20.97±4.41	0.965±0.291	93
*Nor*adrenaline ..	1.63±0.50	0.077±0.035	7

The weight of an adrenal gland was 22.9±5.9 mg./100 g. rat.

Estimates from 10 newborn rats (<24 hours) in groups of 5 each.

Adrenaline .. .. 0.225 : 0.375 μg./gland

*Nor*adrenaline .. .. 0.031 : 0.025 „ „

The rats, weighing 150–200 g., were starved overnight and killed by a blow on the head. Both adrenals from each rat were removed quickly, weighed, and ground with 0.5 ml. 0.15 N–HCl. Ethanol (10 ml.) was added with thorough mixing. After standing 30 min. at room temperature the mixture was centrifuged for 15 min. at 2,000 r.p.m. The clear supernatant was decanted into a 50-ml. Quickfit and Quartz R.B. flask, the residue being washed with 2.5 ml. ethanol. The alcoholic extract was evaporated to dryness *in vacuo* at 55–60° (external temperature). The reddish-brown

residue was leached with 0.75 ml. acid-ethanol (0.1 ml. conc. HCl/100 ml. ethanol) and the extract applied to a paper cylinder. A further extract with 0.5 ml. acid-ethanol was also applied. Each analysis was thus carried out on the pooled glands of a single rat.

The adrenals of newborn rats (less than 24 hr. old) were also analysed, all the glands of five animals being extracted together in 0.5 ml. 0.15 N–HCl.

From the results of these analyses of normal rat's adrenals it is of interest to note that, of the various species of animal for which figures are available, namely the cat, guinea-pig, rabbit, and ox (Schuler and Heinrich, 1949b; Euler, Hamberg, and Purkhold, 1949), the rat has the lowest average percentage of *nor*adrenaline in the pressor amines of the adrenal medulla.

Analysis of a phaeochromocytoma.—Through the kindness of Professor Greenfield and his colleagues of the Royal Victoria Hospital, Belfast, we have had an opportunity of analysing an extract of a phaeochromocytoma by our method. A portion of the tumour was received in 0.1 N–HCl. It was cut into small pieces and reduced to a pulp by grinding in a glass mortar with some of the acid. The addition of a little acid-washed silver sand aided the disintegration. After thorough mixing of the pulp and the acid the mixture was centrifuged at 2,000 r.p.m. for 10 min. The faintly opalescent brown supernatant, *p*H 4 (glass electrode), was stored at 5° overnight. For the determination of the adrenaline and *nor*adrenaline content of the extract some of the fluid was centrifuged at 3,000 r.p.m. for 15 min. to give a clear brown supernatant over a small amount of dark-brown precipitate. Measured quantities (0.05 ml. and 0.10 ml.) of this supernatant were applied to a filter-paper cylinder by means of a graduated 0.2-ml. pipette fitted with a capillary tip, and put through the analytical procedure.

TABLE IV

DETERMINATION OF ADRENALINE AND *nor*ADRENALINE OF A HUMAN ADRENAL MEDULLARY TUMOUR

(5.26 ml. extract ≡ 1 g. tumour)

Vol. of extract analysed ml.	Quantity of amine found	
	Adrenaline μg.	*Nor*adrenaline μg.
0.05	10.0	67
0.10	22.5	133

By calculation from the mean of these assays the tumour contained 7.0 mg. *nor*adrenaline and 1.1 mg. adrenaline/g.

The results for these assays are given in Table IV. It will be seen that the results for *nor*adrenaline in the two samples are in complete agreement while those for adrenaline do not differ by more than 12.5 per cent, which testifies to the accuracy of the method even when relatively large quantities of the amines are present.

In passing it may be mentioned that the proportion of adrenaline to *nor*adrenaline found in this tumour was similar to those recorded in the literature (Holton, 1949; Goldenberg *et al.*, 1949).

Discussion

Adrenaline and *nor*adrenaline in mixtures in simple solution, biological fluids, and tissue extracts can be determined with a high degree of accuracy by the method described above. This method involves the separation of the amines by paper chromatography, elution of the separated amines from the developed chromatogram, and the assay of the separated amines in the eluates by the rat's blood pressure technique. In our hands the procedure has given consistently better and more dependable results than the method of parallel quantitative assays on the isolated uterus and colon of the rat described by Gaddum and Lembeck (1949). In control experiments recoveries of 75–125 per cent have been obtained from pure solutions and from plasma with quantities of adrenaline and *nor*adrenaline ranging from 0.25 μg. to 20 μg. and with either amine in twentyfold excess. It is our belief that the sensitivity, and also to a great extent the accuracy, of the method is limited only by the final assay procedure.

In the course of the development of the method the isolated uterus and colon of the rat and the perfused rabbit's ear (Gaddum, Peart, and Vogt, 1949) had been used for assay purposes, but these were eventually abandoned since they appeared liable to yield erroneous results owing to interference from traces of reagents used during the separation of the amines. The rat's blood pressure was found not to be subject to such interference. Accurate assays of solutions of *nor*adrenaline containing 0.125 μg./ml. and adrenaline solutions containing 0.25 μg./ml. can be made with a sensitive preparation. The ratio of the dose of (−) *nor*adrenaline to the equivalent dose of (−) adrenaline averages 0.25 (0.15–0.75). Rats of either sex weighing 200–250 g. may be used. Smaller animals, though more sensitive, are irregular in their responses, while larger animals are relatively insensitive.

The rat's blood pressure preparation appears to be one of the most sensitive tests for *nor*adrenaline, as little as 0.062 μg. often being determinable. It is thus comparable in sensitivity to the isolated colon of the rat, hitherto regarded as the best biological test for *nor*adrenaline; it has, in addition, the advantage that it is more accurate than the colon preparation. Statistical treatment of assay results by the method of Bliss (1944) and Noel (1945) gave a mean value for λ of 0.096 compared with 0.126 found by Gaddum and Lembeck (1949) for the colon preparation.

It is considered that by the inclusion of the paper chromatographic separation the analytical procedure as a whole gains in objectiveness, since the amines behave like adrenaline and *nor*adrenaline not only pharmacologically but also physicochemically in that they are located on the paper chromatogram at or close to the positions occupied by pure adrenaline and *nor*adrenaline. Further, the chromatography will effect a considerable purification of the extract containing the amines so that the assays will be less liable to interference from other substances in the biological material than when more direct methods of assay are employed.

When the idea of adapting the paper chromatographic separation technique of James (1948) to the quantitative separation of adrenaline and *nor*adrenaline was first considered it was thought that the most likely source of loss would be through oxidation of the amines during the relatively protracted development of the chromatogram. It was with this in mind that the spraying of the paper with ascorbic

acid and the development of the chromatogram in an atmosphere of sulphur dioxide were introduced at the outset. We have not investigated whether either or both these precautions are necessary.

Experiments in which the phenol was removed from the developed chromatograms by heating them at 40° in an oven, through which a stream of nitrogen was passed to sweep out the vapour, showed that such a procedure led to variable results. The detrimental effect of heat on the quantitative recovery of amino-acids from paper chromatograms has also been reported (Fowden and Penney, 1950).

For the application of the method to plasma it is necessary, as a first step, to remove the protein. Some considerable difficulty was experienced in finding a suitable method. It has been reported in the literature that precipitation with trichloroacetic acid or metaphosphoric acid is unsuitable since adrenaline, and presumably *nor*adrenaline, is partly or completely removed from the solution on the protein precipitate (Gaddum and Schild, 1934; Jörgensen, 1945; Lund, 1949). A method of deproteinization used by Folin, Cannon, and Denis (1912) and Frowein (1923) for the extraction of the pressor amines from adrenal glands proved unsatisfactory when applied to plasma. This method entails the denaturation of the protein with acid at 100°, cooling, adjustment of the solution to the isoelectric point of the denatured protein by the addition of sodium acetate, and finally coagulation by heating. The recoveries were variable, and interference was experienced in the assay of the *nor*adrenaline fractions owing probably to the presence of acetate (Bauer and Richards, 1928). Replacement of the acetate by bicarbonate, citrate, or phosphate did not materially improve the recoveries. Also examined but found unsuitable was a modification of a method recently described by Lund (1949) in which adrenaline and *nor*adrenaline are removed from plasma by adsorption on chromatographic alumina (B.D.H.) and eluted therefrom with acid. Of the various methods investigated only the acid-ethanol method which has been described gave consistently good recoveries.

Summary

1. A method is described for the quantitative separation of adrenaline and *nor*adrenaline in pure solution, biological fluids, and tissue extracts.

2. The method depends on the separation of the amines by paper chromatography, elution of the separated amines from the developed chromatogram, and the assay of the separated amines in the eluates by means of the rat's blood pressure preparation.

3. Recovery experiments from pure solution and from plasma showed that the amines can be estimated in quantities as low as 0.25 μg. with an accuracy of 75–100 per cent, this level of accuracy still holding when one of the amines is in twenty-fold excess in a mixture.

4. It is considered that the combination of sensitivity, accuracy, and objectiveness which the procedure possesses makes it capable of a breadth of application not to be found in the hitherto published methods for the determination of adrenaline and *nor*adrenaline in mixtures.

We wish to thank Professor J. H. Gaddum for his interest and encouragement throughout the course of this work. We are also greatly indebted to Miss M. L. Clark for her careful assistance.

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ADDENDUM

A MODIFICATION OF THE CONVENTIONAL MERCURY MANOMETER FOR BLOOD-PRESSURE RECORDINGS

by

N. E. CONDON

Chief Technician, Department of Pharmacology, University of Edinburgh

The mercury manometer used in these experiments is described here because it has been found that the details of the apparatus are especially important in experiments on rats.

The glass portion of the manometer is constructed of thick-walled tubing with an internal diameter of 2–3 mm. The total height of one limb is 24 cm.

The writing point is made of a piece of glass capillary about 9 cm. long and bent in the middle at right angles. The point of one limb writes on the smoked paper and the other limb is in contact with a vertical piece of glass capillary suspended at least 20 cm. above the top of the manometer and adjusted so as to keep the writing point in contact with the paper.

This writing point is attached to the upper end of a length of stainless steel hypodermic needle tubing (about 20 cm. of No. 23 S.W.G.; approximately 0.6 mm. in diameter) passing through a float made of ebonite or polythene tubing. This float fits the glass tubing as closely as possible without sticking, and is mounted about 4 cm. from the lower end of the steel tubing, which is plugged with a piece of wire. The float rests on the surface of the mercury and the lower part of the steel tubing is submerged. This submerged portion is kept central by the float and the usual brass cap on the top of the glass tube through which the upper part of the steel tubing passes. The length of the submerged

part is so adjusted that the upward thrust of the mercury permits the float to rest lightly on the surface of the mercury and to maintain it in that position during all movements of the mercury column. Aluminium wire may be substituted for the steel tubing, in which case the submerged portion of the wire should be well lacquered.

Polythene tubing 3.50 mm. bore is used to connect the B.P. cannula to the manometer. This has several advantages, the main one being that there is practically no loss in the transmission of the impulse from the cannula to the manometer. This is certainly not true when using rubber tubing unless of very heavy wall.

This type of manometer has been found to give very satisfactory records in rat's blood pressure experiments. It is of simple and robust construction, the use of relatively heavy materials for the float and attachments being permitted by the nature of the design.

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INHIBITION OF CHOLINESTERASES BY IRREVERSIBLE INHIBITORS *IN VITRO* AND *IN VIVO*

BY

F. HOBBIGER

From the Department of Pharmacology, Middlesex Hospital Medical School, London, W.1

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Two main types of cholinesterase inhibitors are known: (i) reversible inhibitors, such as the carbamic esters, eserine and neostigmine, and (ii) irreversible inhibitors, such as the alkylphosphates, tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP). The kinetics of cholinesterase inhibition by reversible inhibitors are so complex that it is impossible to correlate cholinesterase inhibition *in vitro* with pharmacological action *in vivo*. The introduction of DFP by McCombie and Saunders (1946) and of TEPP by Schrader (1947) opened therefore a new line for physiological and pharmacological research. Both these compounds have been classified as irreversible inhibitors because neither dilution nor dialysis can restore cholinesterase activity once the enzyme has been inactivated (Brauer, 1948; Aldridge, 1950). Furthermore, the addition of acetylcholine or other substrates to cholinesterase in the presence of these irreversible inhibitors merely stops the progress of enzyme inactivation, but does not, as happens in the presence of eserine or neostigmine, partly reverse it (Burgen, 1949a). Now if these compounds really block cholinesterase irreversibly, and do not alter the speed of resynthesis of the enzyme, the time-course of recovery should be the same for all members of the group; but this is not so (see review by Koelle and Gilman, 1949).

To study this problem experiments have been carried out with TEPP. The effect of this compound *in vivo* has been compared with its inhibitory effect *in vitro*, and the time-course of inhibition has been followed under both these conditions. DFP has been used in these experiments only to help in the interpretation of the experimental results obtained with TEPP.

Materials and Methods

In all experiments male guinea-pigs weighing between 200 and 300 grammes and male albino Wistar rats weighing between 150 and 200 grammes were used. Human serum and red cell esterases were also taken for *in vitro* studies.

The activity of cholinesterase was estimated by the usual Warburg technique at 37° C. (Ammon, 1933). Washed red cells, serum, and homogenized tissues were diluted in $NaHCO_3$ (final concentration 0.025 M) and equilibrated with a 95 per cent N_2 + 5 per cent CO_2 gas mixture; 0.03 M-*dl*-acetyl-β-methylcholine and 0.01 M-benzoylcholine were used as substrates for true and pseudo-cholinesterase respectively.

In experiments where the inhibition of cholinesterase was studied *in vitro* washed red cells and unpurified serum were used as described above, but the tissue homogenates were centrifuged at 2,000 r.p.m. for 20 minutes and only the supernatant taken. This procedure

was adopted because the experiments of this type were designed to determine the sensitivity towards TEPP of cholinesterase itself under conditions where interference by other tissue constituents, especially cell rests, was reduced as far as possible without use of chemical methods for purification. The cholinesterase activity of the supernatant varied between 60 and 80 per cent of the non-centrifuged enzyme preparation.

The enzyme solutions were placed in the main compartment of the vessel, and substrate and inhibitor, each dissolved in 0.2 ml. of 0.025 M-$NaHCO_3$, were placed in separate side-arms. The total volume of fluid was always 3 ml. In all experiments corrections for non-enzymic hydrolysis of the substrate were applied, taking also into account changes of gas volume not due to substrate hydrolysis.

To study the duration of cholinesterase inhibition *in vitro* washed red cells, serum, and the supernatant of centrifuged tissue homogenates were diluted with $NaHCO_3$ (final concentration 0.01 M) or NaCl (final concentration 0.9 g. per 100 ml.). TEPP or DFP was then added in a concentration which produced a marked inhibition of cholinesterase activity and the samples incubated at 30° C. The enzyme activity was estimated at intervals corresponding to the *in vivo* experiments.

In the *in vivo* experiments TEPP or DFP was given by subcutaneous or intraperitoneal injection. The animals were killed after various intervals and the cholinesterase activity estimated by the method described above. Tissue homogenates were used without centrifugation.

The duration of cholinesterase inhibition was also followed in rats by the method described by Burgen (1949b), the threshold concentration of acetylcholine necessary to produce the secretion of red tears (chromodacryorrhoea response) being used as an indicator of the extent of enzyme inhibition.

Most of the experimental figures are expressed as percentages of untreated controls. Enzyme concentrations which produced at least 30 μl. CO_2 in 10 minutes were used in order to obtain significant results.

TEPP and DFP were made up as 5 per cent (v/v) stock solution in dry propylene glycol and from these an aqueous solution was made immediately before use.

Results

Inhibition of cholinesterase in blood and tissues in vivo

Guinea-pig.—TEPP is a very potent inhibitor of cholinesterase, and its action *in vivo* has been investigated in detail by Burgen, Keele, and Slome (1949). In my experiments a dose of 0.05–0.1 mg./kg. produced marked symptoms of the muscarinic type of acetylcholine poisoning such as bradycardia, salivation, and diarrhoea, but only slight nicotinic symptoms, such as muscular twitching. The minimal lethal dose was about 0.1 mg./kg. if the inhibitor was given by subcutaneous injection, and 0.25 mg./kg. after intraperitoneal administration. A dose of 2 mg./kg. of atropine sulphate given half an hour before TEPP reduced the toxicity so that doses of TEPP up to 0.4 mg./kg. did not then cause death, in spite of very marked impairment of neuromuscular transmission. Atropine in such a dose blocked the muscarinic actions of acetylcholine, but had no inhibitory effect on cholinesterase as was shown in control experiments *in vitro*.

In Fig. 1 the reduction in the cholinesterase activity in blood and various organs of the guinea-pig after subcutaneous injection of TEPP is shown. Small doses of the inhibitor, e.g., 0.13 mg./kg., caused a variable degree of cholinesterase inhibition in the various tissues. These differences generally became less with higher concen-

trations of the inhibitor, when a state of saturation was reached. Brain cholinesterase was, however, much less inhibited than that in other organs; this might be due partly to the intervention of the " blood-brain barrier," and partly to the poor lipoid solubility of TEPP.

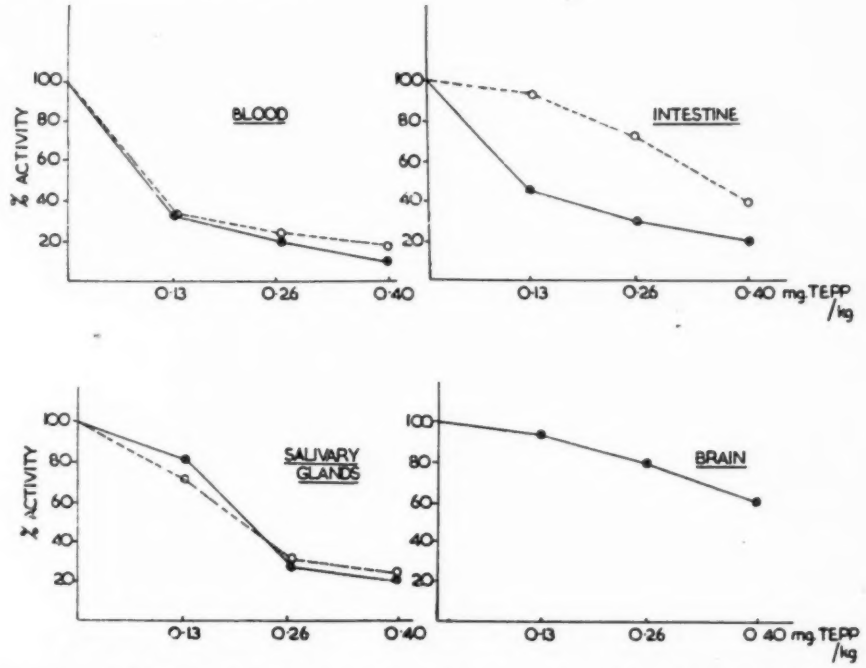

FIG. 1.—*In vivo*. Inhibition of cholinesterase in various tissues in guinea-pigs by TEPP. Ordinates: cholinesterase activity expressed as per cent of the cholinesterase activity of untreated animals. Abscissae: doses of TEPP in mg./kg. The inhibitor was given by subcutaneous injection 30 minutes after injection of 2 mg. atropine sulphate/kg. The animals were killed 30 minutes after the injection of TEPP. Each point represents the average cholinesterase activity of tissues from two animals, two untreated animals serving as controls. ●——● true cholinesterase. O- - - - -O pseudo-cholinesterase.

Rat.—In rats the minimal lethal dose of TEPP was 0.2 mg./kg. for subcutaneous and 0.5 mg./kg. for intraperitoneal injection. The protecting effect of atropine was less marked. As far as the inhibition of cholinesterase by various concentrations of TEPP was concerned the result was similar to that obtained in guinea-pigs, except that the brain was inhibited to a somewhat greater extent.

In vitro *inhibition of cholinesterases prepared from blood and tissues*

Guinea-pig.—The next step was to correlate these *in vivo* findings with the inhibition achieved by TEPP *in vitro*. The results of experiments designed for this purpose are summarized in Table I.

The concentrations of TEPP which produce a 50 per cent inhibition in 20 minutes at 37° C. in equiactive enzyme preparations (for a given substrate) were chosen for comparison. As can be seen from the results in Table I the true cholinesterases in red cells, brain, and salivary glands were inhibited to the same extent by roughly

TABLE I

INHIBITION OF CHOLINESTERASES *in vitro*

Guinea-pig enzyme preparations		Concentrations of TEPP required to produce 50% inhibition in 20 min. at 37° C.	
		True cholinesterase Substrate: 0.03 M-acetyl-β-methylcholine	Pseudo-cholinesterase Substrate: 0.01 M-benzoylcholine
Red cells	0.2 ml.	7.8×10^{-9}	—
	0.4 ml.	8.7×10^{-9}	—
Serum	0.3 ml.	—	4.5×10^{-9}
	0.6 ml.	—	1.25×10^{-8}
Brain	50 mg.	5.3×10^{-9}	—
	100 mg.	6.0×10^{-9}	—
Salivary glands	7 mg.	—	6.4×10^{-10}
	14 mg.	—	1.0×10^{-9}
	80 mg.	5.2×10^{-9}	—
	160 mg.	6.2×10^{-9}	—
Intestine	65 mg.	—	2.7×10^{-8}
	130 mg.	6.4×10^{-8}	6.1×10^{-8}
	260 mg.	7.6×10^{-8}	—

equal concentrations of TEPP. Serum esterase was less sensitive to TEPP than the pseudo-cholinesterase of salivary glands. If the amount of enzyme was doubled higher concentrations of TEPP were necessary to produce the same degree of inhibition. This was particularly marked with the pseudo-cholinesterases.

The cholinesterases obtained from the intestine behaved quite differently, much higher concentrations of the inhibitor being necessary to achieve a 50 per cent inhibition. Such a difference might arise in the following ways:

(*a*) The enzymes in the different organs, though all classified as true or pseudo-cholinesterases, might differ in some respects, e.g., in their sensitivity to inhibition by TEPP.

(*b*) The inhibitor might be destroyed before combination with cholinesterase; this process of destruction might occur at different speeds in various organs.

(*c*) The inhibitor might combine not only with cholinesterase but also with other tissue constituents; such an " unspecific " combination could reduce the concentration of TEPP available for cholinesterase inhibition to different degrees in different organs.

A closer analysis of these three points gave the following results:

(*a*) The terms " true cholinesterase " and " pseudo-cholinesterase," as defined by Mendel and his co-workers (1943, 1944), are strictly applicable to the enzymes mentioned in Table I. For example, the pseudo-cholinesterase preparations from serum, salivary glands, and intestine showed identical properties, in that they all split acetylcholine about twice as fast as benzoylcholine, and that higher concentrations of acetylcholine had no depressant effect on the enzyme activity (taking

into account the effect of true cholinesterase which was present in these unpurified enzyme preparations). The true cholinesterase preparations from brain, salivary glands, and intestine behaved just like human red cells when *dl*-acetyl-β-methylcholine was used as substrate in various concentrations (Augustinsson, 1948).

Thus there is nothing to suggest that any significant differences exist among the true and pseudo-cholinesterases derived from the various sources, and there is no direct evidence that the enzymes in the preparations from different tissues are differentially inhibited by TEPP.

(*b*) In order to study the capacity of tissue homogenates to destroy TEPP it was first necessary to measure the rate of spontaneous hydrolysis of this substance in solution. TEPP, therefore, was made up in a concentration of 3×10^{-7} in 0.01 M-$NaHCO_3$ and incubated at 37° C. for varying periods. The activity of the inhibitor was reduced by 50 per cent after 3½ hours, and after 24 hours no significant anti-cholinesterase effect could be detected. Higher dilutions of TEPP showed a similar breakdown.

In two experiments TEPP in a concentration of 2×10^{-9} was incubated with 100 mg. intestine at 37° C. for 20 minutes. The mixture was then brought to a *p*H of 3.0–4.0, heated for 3 minutes at 95° C. (to destroy all cholinesterase activity), then cooled and the *p*H adjusted to 7.2. It was found that 12 and 20 per cent respectively of the original amounts of TEPP had disappeared as a result of these procedures. Control experiments showed a disappearance rate of about 5 per cent. The concentration of TEPP required to produce 50 per cent inhibition of the pseudo-cholinesterase in 65 mg. intestinal homogenate in 20 minutes was a hundred times that required to produce 50 per cent inhibition of the enzyme in 0.2 ml. of human serum in the same time. The relatively small increase in rate of breakdown of TEPP in the presence of intestinal tissue can only explain a very small fraction of this observed hundredfold difference.

(*c*) The experiments under (*a*) and (*b*) suggest that the small inhibitory effect of TEPP on the enzyme preparations obtained from intestine might be due to com-

TABLE II

EFFECT OF NON-SPECIFIC COMBINATION ON THE APPARENT SENSITIVITY OF UNPURIFIED ENZYME PREPARATIONS TO TEPP

Source of enzyme	Substrate	Concentration of TEPP which produced 50% inhibition in 20 min.
0.2 ml. human serum	Benzoylcholine	2.2×10^{-10}
0.04 ml. human red cells ..	Acetyl-β-methylcholine	3.2×10^{-9}
65 mg. guinea-pig intestine ..	Benzoylcholine	2.7×10^{-8}
65 mg. guinea-pig intestine + 0.2 ml. human serum	Benzoylcholine	3.0×10^{-8}
65 mg. guinea-pig intestine + 0.04 ml. human red cells ..	Acetyl-β-methylcholine	5.3×10^{-8}

bination of a large amount of inhibitor with other tissue constituents before it comes in contact with the cholinesterase. Experiments were therefore performed to study this possibility. From Table II it will be seen that a concentration of 2.2×10^{-10} TEPP is required to inhibit serum pseudo-cholinesterase by 50 per cent in 20 minutes. A concentration of 2.7×10^{-8} TEPP is required to inhibit a homogenate of 65 mg. intestine to the same degree. This difference in sensitivity is about a hundredfold. Since equiactive concentrations of both enzyme preparations were chosen, a concentration of roughly 2.5×10^{-9} TEPP would be expected to give a 50 per cent inhibition for a mixture of both. Actually 3×10^{-8} TEPP is necessary to produce this effect. Table II also shows that a similar result was obtained when human red cells were used instead of serum.

Similar results have been obtained with other enzyme preparations of the guinea-pig and with rats.

Duration of cholinesterase inhibition by TEPP in vivo

Most of the work on the actions of cholinesterase inhibitors *in vivo* has been concerned with the recovery of enzyme activity in blood. Only few authors have extended their work to the study of other organs. The only available information about the alkylphosphates relates to the duration of enzyme inhibition in blood, brain, and muscle (Freedman, Willis, and Himwich, 1949; Mazur and Bodansky, 1946). This work has therefore been extended, and the results are given in Fig. 2. It will be seen that after inhibition by TEPP cholinesterase reactivation *in vivo* proceeds rather rapidly, and at about the same rate in blood, brain, and intestine; in salivary glands, however, it is at least three times as fast.

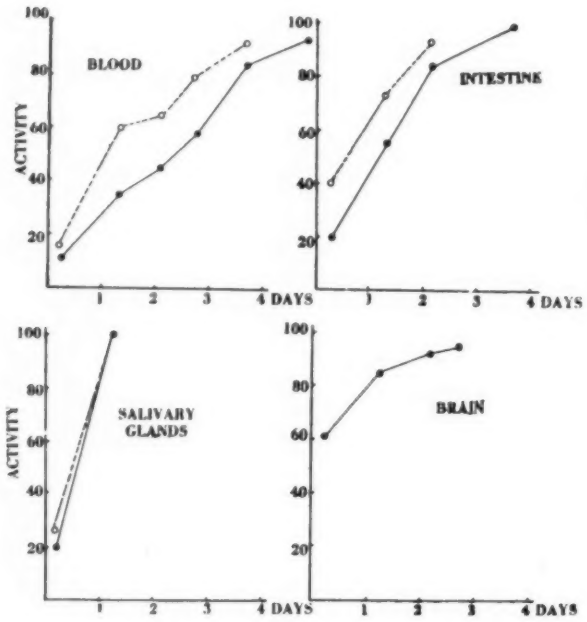

FIG. 2.—Duration of cholinesterase inhibition in guinea-pigs after subcutaneous injection of 0.4 mg. TEPP/kg.; 2.0 mg. atropine sulphate was given 30 minutes before the inhibitor. Ordinates: cholinesterase activity as per cent of control. Abscissae: days of observations. ●——● true cholinesterase. O- - - - - O pseudo-cholinesterase.

The difference in duration of action between DFP and TEPP *in vivo* was studied by means of the chromodacryorrhoea response in rats (Burgen, 1949b). Fig. 3 shows that, starting with comparable degrees of cholinesterase inhibition, recovery of normal sensitivity to acetylcholine occurred in six days after TEPP, but that after DFP only 50 per cent recovery occurred in ten days. If it is accepted that neither

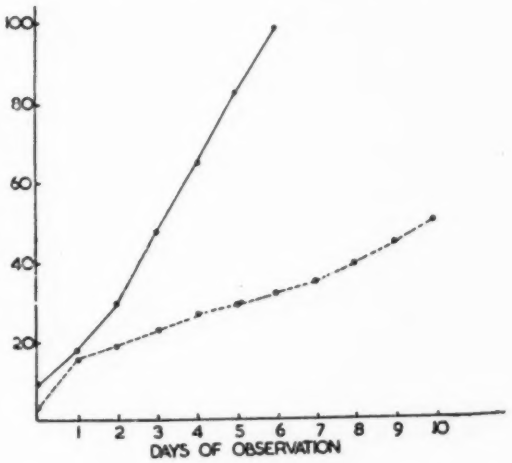

FIG. 3.—Duration of cholinesterase inhibition by DFP and TEPP in rats *in vivo* (chromodacryorrhoea response). 0.4 mg. TEPP/kg. and 4.0 mg. of DFP/kg. respectively were given by intraperitoneal injection. Ordinates: the amount of acetylcholine (as per cent of that in three tests before injection of inhibitor) which had to be given to stimulate the secretion of red tears. Abscissae: days of observations. ●———● acetylcholine threshold after TEPP. O- - - - -O acetylcholine threshold after DFP.

DFP nor TEPP has any influence on cholinesterase resynthesis, this difference in enzyme recovery can only be explained by assuming that the TEPP-enzyme complex is not stable, or that the cholinesterase molecule (or another enzyme) can destroy TEPP after this complex has been formed. It is not possible to study this question further *in vivo*, so *in vitro* experiments were carried out to obtain further evidence. TEPP and DFP are both unstable in aqueous solutions, and, if red cells, serum, or tissue homogenates are incubated with an amount of TEPP or DFP sufficient to produce a marked reduction of esterase activity, any inhibitor not combined with active enzyme centres will be completely inactivated after incubation for twenty-four hours; it is thus possible to observe reactivation of the enzyme without previous dialysis. The activity of the control samples remained relatively constant during the period of observations, not more than 10 per cent loss of activity being observed. The results of such experiments are shown in Tables III and IV. It will be seen in Table III that in the presence of DFP no enzyme reactivation occurred *in vitro*. If TEPP, however, was used as an inhibitor (Table IV) the inhibition of cholinesterase

TABLE III

GUINEA-PIG CHOLINESTERASES INCUBATED WITH 10^{-7} DFP FOR VARIOUS PERIODS AT 30° C.

Source of enzyme	Activity as per cent of control after			
	1 hr.	24 hr.	48 hr.	72 hr.
Brain	22	8	7	8
Red cells	18	2	5	4

TABLE IV

REACTIVATION OF CHOLINESTERASES FROM VARIOUS SOURCES AFTER INHIBITION BY TEPP *in vitro*

Source of enzyme	Activity as per cent of control after incubation at 30° C. for				Activity expressed in μl. CO_2 produced in 10 min.	
	1 hr.	24 hr.	48 hr.	72 hr.	At beginning of experiment	After 72 hours' incubation
Guinea-pig:						
(1) 0.3 ml. red cells ..	15	33	49	65	7.1	33.2
(2) 100 mg. brain ..	5	22	45	58	4.4	46.4
(3) 0.3 ml. serum ..	8.5	31	43	55	3.1	19.8
(4) 20 mg. salivary glands	13	57	75	—	11.6	60.0 (after 48 hours' incubation)
Human blood:						
(5) 0.06 ml. red cells ..	2	26	42	55	0.96	24.75
(6) 0.2 ml. serum ..	11	14	17	16	5.1	7.3

proved to be reversible (except with human serum esterase). The reactivation was a real one and not due to a slowing down of enzyme inactivation as can be seen from Table IV. This confirms the results of Grob and Harvey (1949), who showed that the inhibition of red cell esterase by TEPP was reversible for the first twenty-four hours. Enzyme preparations obtained from rats, and human red cell esterase, behaved like those from guinea-pigs. The process of recovery was always fastest with homogenates of salivary glands, a finding which correlates well with the *in vivo* experiments. Mg^{++} ions activated this recovery process and Cu^{++} ions had an inhibitory effect. The enzyme reactivation is not related to bacterial action, because neither thymol nor other bacteriostatic agents had any significant influence.

If red cells, or one of the other enzyme preparations used for these experiments, were incubated in the presence of TEPP, and after 24 or 48 hours the same or a different type of cholinesterase was added, the process of enzyme recovery was not enhanced.

Discussion

The experiments here described have shown that TEPP inhibits both true and pseudo-cholinesterase activity *in vitro* to varying degrees in different tissues. The factors which influence the degree of inhibition *in vitro* are:

1. The concentration of inhibitor.

2. With irreversible inhibitors, the concentration of enzyme (Ackermann and Potter, 1949; Bain, 1949; Augustinsson and Nachmansohn, 1949).

3. Reduction in the effective concentration of inhibitor by the action of tissue constituents other than cholinesterase. This factor has been demonstrated by the finding that equiactive enzyme preparations from blood or tissues of the same animal differ widely in their sensitivity to TEPP. There is no evidence that any significant breakdown of TEPP occurs before it combines with the tissue constituents (including cholinesterase), and the reduction in the effective concentration of the inhibitor is most probably due to non-specific combination with tissue components (probably proteins) other than cholinesterase. Such a mechanism might

be of importance in relation to other anticholinesterases, and might account for the relative insensitivity of frog brain esterase to eserine (Hawkins and Mendel, 1946). Mazur and Bodansky (1949) concluded from their experiments with DFP that non-specific binding by proteins or other tissue constituents is of no importance in relation to cholinesterase inhibition, but their results were obtained on heat-denatured brain mixtures only and are therefore of doubtful significance.

The conditions of cholinesterase inhibition *in vivo* must take account of still other factors, such as distribution via the blood stream, passage from blood to tissue (especially important in the case of the brain), localization of cholinesterase in the tissue, and physical properties, such as the lipoid solubility of the inhibitor. With these points in mind, we are not surprised to find that there are discrepancies between the *in vitro* and *in vivo* measurements of the activity of TEPP.

Recovery of cholinesterase activity

The time-course of recovery of cholinesterase activity depends on the nature of the inhibitor used (Koelle and Gilman, 1949). With DFP the inhibition is long-lasting *in vivo* and irreversible *in vitro* (Fig. 3; Table III). With TEPP the duration of cholinesterase inhibition *in vivo* is much shorter than with DFP, and the action *in vitro* is reversible (Figs. 2 and 3; Table IV). The *in vitro* experiments have shown that the reactivation after TEPP is due either to dissociation of the enzyme-inhibitor complex or to destruction of the inhibitor by cholinesterase; there is no evidence for the intervention of a second enzyme, such as phosphatase. Mg^{++} ions have an activating effect and Cu^{++} ions slow down the process of enzyme reactivation. The true and pseudo-cholinesterases of blood, brain, intestine, and salivary glands of guinea-pigs and rats, and human red cells, all show reactivation after inhibition by TEPP. *In vivo* some differences are seen and salivary glands always recover first, in spite of the marked initial inhibition (Fig. 2).

Human serum esterase is exceptional. No significant recovery from inhibition by TEPP was observed *in vitro*, and *in vivo* little difference exists between the time-course of recovery after inhibition by TEPP and DFP respectively (Freedman, Willis, and Himwich, 1949; Mazur and Bodansky, 1946).

Summary

1. The degree of *in vivo* inhibition of cholinesterase in guinea-pigs and rats produced by small concentrations of TEPP varies widely among different tissues. With higher concentrations of the inhibitor these differences disappear almost completely, brain being an exception.

2. The inhibition of cholinesterase by TEPP *in vitro* depends on the concentration of this inhibitor, the concentration of enzyme, and the amount of inhibitor which combines with tissue constituents other than cholinesterase (e.g., proteins). The correlation between *in vivo* and *in vitro* experiments is discussed.

3. The inhibition of cholinesterase by DFP *in vitro* is irreversible. The inhibition by TEPP *in vitro* is reversible, except with the enzyme of human serum. This reactivation is thought to be due either to a dissociation of the enzyme-inhibitor

complex or to destruction of the inhibitor after combination with cholinesterase. Enzyme recovery is favoured by Mg^{++} and hindered by Cu^{++}; it proceeds at different speeds in enzyme preparations from different organs.

I should like to thank Dr. C. A. Keele for his encouragement during the course of this work, and especially for his help in the preparation of the manuscript.

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SUSCEPTIBILITY TO VENTRICULAR FIBRILLATION DURING CHLOROFORM AND CYCLOPROPANE ANAESTHESIA

BY

D. E. HUTCHEON

From the Department of Pharmacology, University of Toronto

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The experimental production of ventricular fibrillation during chloroform anaesthesia has been investigated often on several species of animals. There are, however, few studies in which both chloroform and cyclopropane have been used and compared in the same experimental series. In this study the susceptibility of various species of animals to the production of ventricular fibrillation by adrenaline under chloroform and cyclopropane anaesthesia was investigated. The effect of depth of anaesthesia under cyclopropane was a'so studied in cats and dogs and a few experiments were done on the influence of morphine and of Dibenamine (N: N-dibenzyl-β-chloroethylamine) on the response to adrenaline in guinea-pigs and dogs.

METHOD

Chloroform anaesthesia was induced and maintained by bubbling air through chloroform into a cylinder covering the head of the animal. By changing the rate of flow of the air bubbled through the chloroform, the concentration of the vapour could be controlled easily. Cyclopropane was administered from a spirometer; soda lime removed carbon dioxide from the system.

Small doses of adrenaline were injected during light and deep anaesthesia, the stage of anaesthesia being judged by the presence or absence of the corneal reflex, by the rate and depth of respiration, and by the amount of relaxation. Light anaesthesia comprised the lower part of Stage II and first plane of Stage III, and deep anaesthesia the fourth plane of Stage III and the upper part of Stage IV. Changes in the heart rate and rhythm were observed by means of a stethoscope or an electrocardiograph which consisted of a vacuum tube amplifier operating an ink-writing oscillograph. In the smaller species, if respirations ceased and the heart sounds became inaudible, an autopsy was performed at once and the state of the heart and lungs was noted. A diagnosis of ventricular fibrillation was made by the observation of rapid, tremulous contractions of minute areas of the ventricular myocardium.

RESULTS

In guinea-pigs adrenaline (0.1 mg. per kg. intramuscularly) caused ventricular fibrillation during light chloroform but not during light cyclopropane anaesthesia (Table I). Smith (1949) found that adrenaline did not lead to ventricular fibrillation in guinea-pigs under deep cyclopropane. With both anaesthetics increased depth of anaesthesia caused bradycardia, missed beats, and heart block, while decreased

depth of anaesthesia restored the cardiac rate and rhythm to normal. Death from overdosage was due to respiratory failure, since the breathing ceased while the heart was still beating vigorously. The dose of adrenaline employed was not considered to be toxic to the unanaesthetized guinea-pig, because doses up to 1.0 mg. per kg. were not lethal. However, adrenaline in doses of 2.0 mg. per kg. caused death in three of six guinea-pigs, death being due in two animals to ventricular fibrillation and in one to acute pulmonary oedema.

Adrenaline did not cause ventricular fibrillation in rats or rabbits under light or deep cyclopropane or chloroform anaesthesia (Table I).

TABLE I

OCCURRENCE OF ADRENALINE-INDUCED VENTRICULAR FIBRILLATION IN VARIOUS SPECIES DURING CHLOROFORM AND CYCLOPROPANE ANAESTHESIA

Species	Adrenaline mg. per kg	Chloroform		Cyclopropane	
		Number	V.F.	Number	V.F.
Rats	0.2 i.m.	12	0	10	0
Guinea-pigs	0.1 i.m.	12	4	14	0
Rabbits	0.02 i.v.	10	0	5	0
Cats	0.04–0.08 i.v.	12	3	12	0
Dogs (light)	0.01–0.02 i.v.	10	5	13	0
,, (deep)	0.01–0.02 i.v.			8	5

In three of twelve cats during light chloroform anaesthesia, ventricular fibrillation was induced by the intravenous injection of adrenaline (0.04 to 0.08 mg. per kg.). A transient tachycardia developed in all the other cats after the adrenaline. Spontaneous ventricular extrasystoles occurred frequently during cyclopropane anaesthesia. Of thirteen cats anaesthetized with cyclopropane, one died of ventricular fibrillation during the induction period. Adrenaline (0.04–0.08 mg. per kg.) injected intravenously into the remaining cats during light and deep cyclopropane resulted in a tachycardia which never progressed to fatal ventricular fibrillation.

Of ten dogs injected intravenously with 0.01–0.02 mg. adrenaline per kg. during light chloroform anaesthesia, five died of ventricular fibrillation (Fig. 1). A short run of ventricular tachycardia preceded the fibrillation. Of the five animals that survived, four developed a multiple focus ventricular tachycardia (Fig. 1) and one developed an auricular tachycardia after adrenaline; these rapid cardiac rates lasted from 26 to 54 seconds. Intravenous injections of 0.01–0.02 mg. adrenaline per kg. into thirteen dogs during light cyclopropane anaesthesia resulted only in ventricular extrasystoles or a transient ventricular tachycardia. However, when eight of these dogs were anaesthetized to the stage of slow shallow respirations and were then injected with the same dose of adrenaline, five developed ventricular fibrillation and died (Fig. 1).

Effect of morphine.—In guinea-pigs, the intraperitoneal injection of morphine sulphate (10 mg. per kg.) 15 min. before induction with chloroform did not prevent the development of ventricular fibrillation. Of ten animals so treated and given 0.1 mg. adrenaline per kg. intramuscularly five died of ventricular fibrillation.

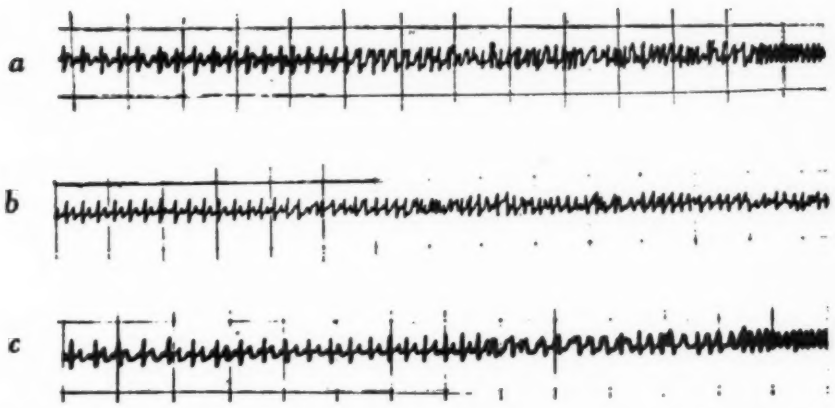

FIG. 1.—Electrocardiograms (lead IV) of dogs injected intravenously with adrenaline (0.02 mg. per kg.). Adrenaline induced in (*a*) ventricular fibrillation during light chloroform, in (*b*) multiple focus ventricular tachycardia during light cyclopropane, and in (*c*) ventricular fibrillation during deep cyclopropane. Time intervals 1 sec.

Effect of dibenamine.—Eight dogs were injected intravenously with dibenamine (12–30 mg. per kg.) and lightly anaesthetized with chloroform. When adrenaline (0.01 mg. per kg.) was injected two died of ventricular fibrillation while the other animals developed a transient tachycardia.

DISCUSSION

It is believed that ventricular fibrillation is likely to develop when impulses pass into the myocardium containing some fibres in a refractory state. Such fibres may be excited to contract a little later when an impulse reaches them by a more devious pathway than normal. If conditions are suitable a circus movement in the ventricle may become established. When the myocardium contains localized blocks to the transmission of the impulses, and the frequency of the impulses is high, the conditions are favourable to the production of fibrillation. Garb and Chenoweth (1948) found that the excitability of the papillary muscle of the ventricle is markedly decreased by chloroform in contrast to ether, which decreases it only slightly, so that during chloroform anaesthesia it is conceivable that localized blocks in the myocardium may occur. Robbins and Baxter (1937) believed that the cardiac irregularities that occur during deep cyclopropane anaesthesia were due to anoxaemia and not to cyclopropane, but Lee, Orth, Wangeman, and Meek (1943) found that arrhythmias developed even in the presence of a normal oxygen saturation of the blood. It appears, therefore, that a high concentration of cyclopropane also depresses the myocardium and results in localized blocks to the transmission of impulses.

Adrenaline is also an important aetiological factor in the precipitation of ventricular fibrillation, because it causes this arrhythmia not only during chloroform and cyclopropane anaesthesia but, in larger doses, in normal unanaesthetized guinea-pigs. Cardiac sympathectomy (Allen *et al.*, 1945) and adrenergic blocking agents, e.g., dihydroergotamine methanesulphonate (DHE 45) and dibenamine, are said to prevent ventricular tachycardia and fibrillation during cyclopropane anaesthesia

(Orth and Ritchie, 1947; Nickerson and Nomaguchi, 1949). In this work dibenamine was tried, and it was not entirely successful in preventing adrenaline-induced ventricular fibrillation in dogs under chloroform.

Summary

1. Under light chloroform anaesthesia the injection of adrenaline resulted in ventricular fibrillation in guinea-pigs, cats, and dogs. Under cyclopropane, adrenaline induced ventricular fibrillation only in dogs during deep anaesthesia.

2. In rabbits, adrenaline did not lead to ventricular fibrillation during chloroform or cyclopropane anaesthesia.

3. Dibenamine did not always protect dogs under chloroform from ventricular fibrillation.

The author takes pleasure in expressing his thanks to Dr. J. K. W. Ferguson and Dr. G. H. W. Lucas for their advice and criticism. This work was carried out during the tenure of a Medical Research Fellowship awarded by the National Research Council of Canada.

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ESTIMATION AND PHOTOLABILITY OF SOME THIOSEMICARBAZONES

BY

A. SPINKS

From Imperial Chemical Industries Limited, Research Laboratories, Hexagon House, Blackley, Manchester, 9

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The author (1949) has already described a spectrophotometric method of estimating three benzaldehyde thiosemicarbazones in blood, among them the highly active *p*-ethylsulphonyl derivative (8388, "Berculon B,"* Hoggarth, Martin, Storey, and Young, 1949). Since then it has become known that the *p*-acetylamino derivative (I, " Conteben," 9311, " Berculon A,"* " Tibione ") has received extensive therapeutic trials in Germany, and numerous reports, usually claiming varying degrees of

I

effectiveness, have appeared. These reports and other, previously unpublished, information have recently been summarized by Hinshaw and McDermott (1950). This paper presents modifications of the earlier method that permit simultaneous estimation of berculon A and the corresponding unacetylated amine 6198 (Hoggarth, Martin, Storey, and Young, 1949), and other changes that raise its sensitivity. Increased sensitivity is necessitated by the low doses of berculon A used clinically. A dose of 200 mg. daily gives a maximum concentration of about 0.1 mg./100 ml. blood (Martin and Spinks, unpublished data).

The photolability of the thiosemicarbazones is also described: it cannot be disregarded if an accurate analysis is to be made.

Experimental Section

Estimation of thiosemicarbazones

p-Acetylaminobenzaldehyde thiosemicarbazone (berculon A) can be hydrolysed to a diazotizable amine, and a colorimetric method based on this property has been described, although not in detail, by Behnisch, Mietzsch, and Schmidt (1950). This method was studied independently, but was abandoned because the thiosemicarbazone group proved to be labile under the conditions of hydrolysis used, and because a spectrophotometric procedure seemed to offer greater specificity and almost equal sensitivity. When it was found that the therapeutic concentrations of berculon A were very low, attempts were made to develop an alternative method by converting the drug to a coloured phenylhydrazone, for example, by acid hydrolysis in the presence of 2: 4-dinitrophenylhydrazine. It was hoped eventually to use a fluorescent

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hydrazine. So far no useful method has been developed, but the possibility is still being studied. Another type of procedure that has been tested is the conversion of the thiosemicarbazones into stable metallic salts by reaction with a known amount of a metallic ion, usually bivalent mercury, followed by colorimetric estimation of the excess mercury with dithizone. The thiosemicarbazone-mercury complexes, although closely related structurally to the red dithizone-mercury complexes, are colourless. Berculon A can be estimated by this technique, but the sensitivity is low.

Only the spectrophotometric methods will be described in detail. There are three of these. The spectrophotometric method of estimating berculon B (Spinks, 1949) was first modified to allow simultaneous estimation of berculon A and *p*-aminobenzaldehyde thiosemicarbazone, which could possibly be formed *in vivo* by deacetylation. Although later work showed that this conversion could occur in mouse and dog only to a slight extent, if at all, the method will be described because species differ greatly in the extent to which acetylation or deacetylation of sulphonamides predominates (Krebs, Sykes, and Bartley, 1947), and others might be able to deacetylate berculon A more readily. The second method is almost identical with that previously described for the estimation of berculon B (Spinks, 1949); it is used after the administration of high doses to species in which deacetylation has been shown not to occur. The third method is suitable for the estimation of 0.5 to 5.0 μg. of drug in 3.0 ml. of blood.

All methods must be performed in weak unfiltered tungsten light, of about the strength that can be used in photographic work for handling contact paper.

Method I. Simultaneous estimation of p-*aminobenzaldehyde thiosemicarbazone* (6198) *and* p-*acetylaminobenzaldehyde thiosemicarbazone* (*berculon A*)

Details of technique and reagents are the same as those prescribed for the estimation of berculon B (Spinks, 1949), with the addition of 1 mg./100 ml. standard aqueous solutions of 6198 and berculon A, freshly prepared by diluting 50 mg./100 ml. solutions in methanol. These solutions must be stored in the dark.

Procedure.—Shake 2 ml. of blood and 2 ml. of 0.2 M-disodium hydrogen phosphate with 40 ml. of chloroform (*B.P.*) for 5 minutes, and filter the extract into a 10-cm. cell. Read the optical densities at 320 and 342 mμ against a blank (extract of 2 ml. of water): let the readings be u_1 and u_2 respectively. Also read the optical densities (a_1 and a_2) at the same wavelengths of a standard prepared by extracting 2 ml. of a 1 mg./100 ml. solution of 6198, and those (b_1 and b_2) of a corresponding standard extract of berculon A. Let the concentrations of 6198 and berculon A in the unknown be x and y mg./100 ml. respectively. Then, assuming linearity of the concentration-optical density graphs, it follows that

$$u_1 = xa_1 + yb_1 \text{ and } u_2 = xa_2 + yb_2$$

$$\text{hence} \quad x = \frac{b_1u_2 - b_2u_1}{a_2b_1 - a_1b_2} \text{ and } y = \frac{a_2u_1 - a_1u_2}{a_2b_1 - a_1b_2}$$

Notes.—This method is based on one previously described for the simultaneous colorimetric estimation of *p*-methylthioaniline and its metabolite, *p*-methylsulphonylaniline (Spinks, 1948). It depends on dissimilarity of the absorption spectra of 6198 and berculon A (Fig. 1) and on linearity of the concentration-optical density graphs of both compounds at both wavelengths. This was established directly and is also demonstrated by the recovery of the two compounds shown in Table I. Neither was recovered quantitatively from

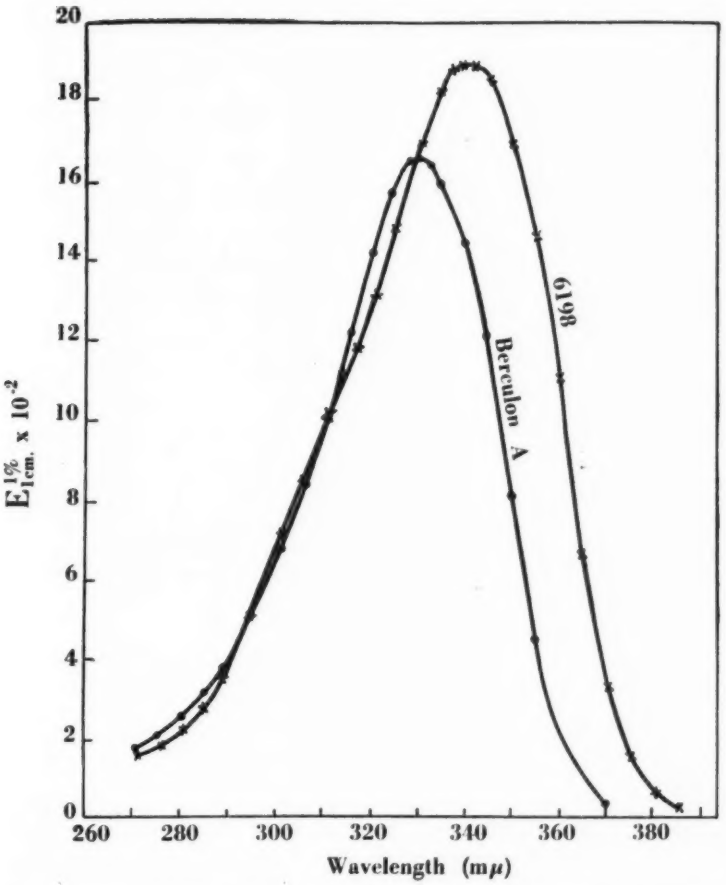


FIG. 1.—Absorption spectra of 6198 and berculon A in chloroform.

blood, and results must therefore be multiplied by a recovery factor. The blank in normal blood is low, of the order of 0.05 to 0.1 mg./100 ml. for berculon A, and —0.05 to —0.01 mg./100 ml. for 6198; the negative sign arises from the fact that the ratios of optical densities, $d320/d342$, are about 1.4, 1.05, and 0.69 for normal blood, berculon A, and 6198 respectively. The apparent concentration of 6198 found in normal blood before dosing is therefore added to the values obtained after dosing.

Concentrations of the two compounds found in mouse blood after the administration of each alone are shown in Figs. 2 and 3. 6198 was not detected with certainty after the administration of berculon A, and was rapidly acetylated to give berculon A when it was itself administered. Indeed, 100 mg. 6198/kg. gave higher concentrations of berculon A than 500 mg. of the latter did. Presumably berculon A is poorly absorbed and 6198 well absorbed. Compound 6198 is more toxic than berculon A and its administration in place of berculon A is probably inadvisable.

The concentrations of berculon A in Fig. 3 are about a quarter of those given by berculon B under the same conditions (Spinks, 1949). A ratio of this order has been established also for other doses in mice and for the dog (cf. Fig. 5) and monkey (Francis, Spinks, and Stewart, 1950).

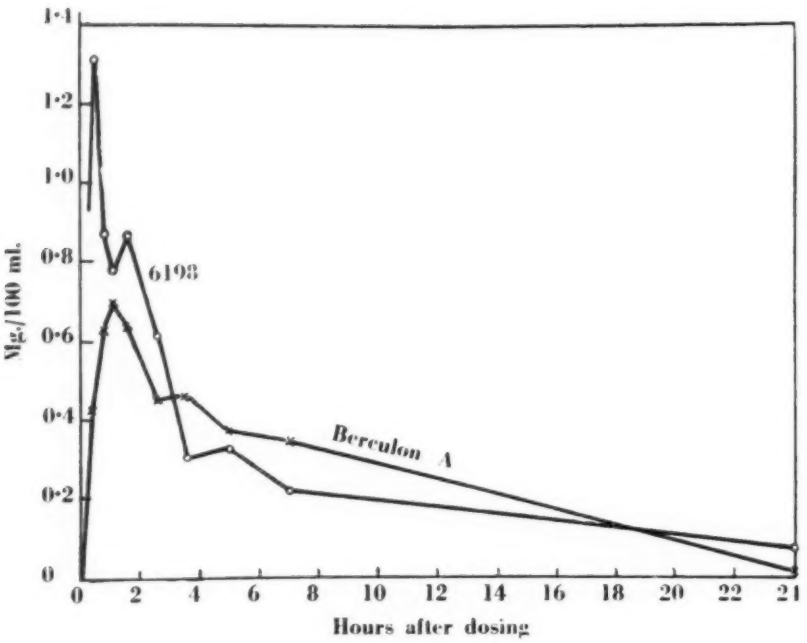


FIG. 2.—Concentrations of 6198 and berculon A in the blood of mice receiving 100 mg. 6198 per kg.

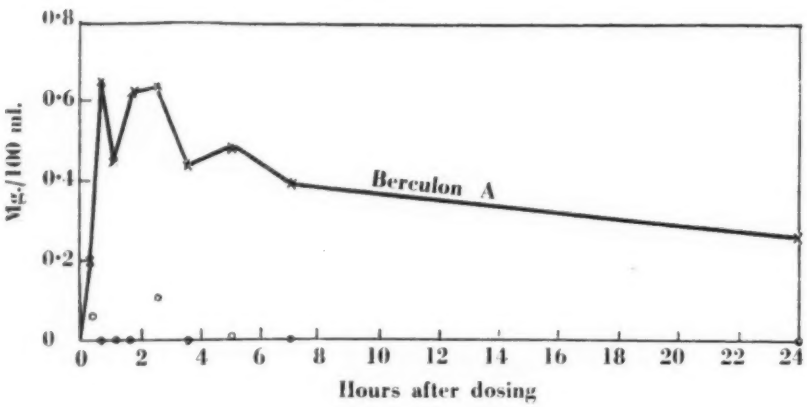


FIG. 3.—Concentrations of 6198 (O) and berculon A (×) in the blood of mice receiving 500 mg. berculon A per kg.

Method II. Estimation of berculon A alone in high concentration

Reagents and conditions are as prescribed for the estimation of berculon B (Spinks, 1949) with the addition of a standard solution of berculon A. Extracts are read at λ_{max} 330 mμ.

Notes.—The recovery of berculon A alone from blood is given in Table II. The mean recovery did not differ significantly from that given in Table I. This method is suitable for estimating 5 μg. and more of berculon A in 2 ml. of blood.

Method III. Estimation of berculon A in low concentration

Three ml. of blood are shaken with 40 ml. of chloroform for 5 minutes. The extract is filtered into a 10-cm. spectrophotometer cell, and the optical density is read every 5 mμ from 280 to 380 mμ. The absorption spectrum is drawn (Fig. 4), using the largest con-

TABLE I

RECOVERY OF BERCULON A AND 6198 FROM BLOOD (2 ML.) BY SIMULTANEOUS SPECTROPHOTOMETRIC ESTIMATION AT λ_{max} 320 AND 342 mμ
(Method I)

Added (μg.)		Found (μg.)		Recovery (%)	
6198	Berculon A	6198	Berculon A	6198	Berculon A
0	0	(0)	(1.10)	—	—
20	0	16.2	(3.56)	81	—
0	20	(0.72)	18.3	—	86
4	4	3.66	4.72	91	90
10	10	9.03	8.83	90	77
10	20	8.38	19.0	84	89
20	10	17.8	9.13	89	80
0	0	−0.41	2.14	—	—
4	4	3.28	5.44	92	82
12	12	9.70	13.4	84	94
20	20	16.3	17.9	83	79
28	28	26.3	23.1	95	75
40	40	34.0	33.4	86	78
20	0	18.1	(2.10)	92	—
0	20	(1.50)	16.7	—	73
			Mean	88	82
			Limit of error of mean (P = 0.05)	±3.0	±4.5

venient scale. If berculon A is present the curve will show a hump, with a maximum near 330 mμ. On either side of this hump there will be a point of sharp curvature. Join the points of sharp curvature by a straight line meeting the curve tangentially at both points. Then if the absorption spectrum of a normal extract is approximately linear (Fig. 4) the part of the curve above the tangent will not include any absorption due to normal constituents. Record the optical density increment (a) above the tangent at 330 mμ. Now note the wavelengths at which the tangent meets the curve. Join the same wavelengths on the absorption spectrum of an extract of 3 ml. of a 0.1 mg./100 ml. solution of berculon A by a straight line. Let the density increment at 330 mμ above the line be a' and that below b'. Then the approximate optical density of berculon A in the unknown blood is $F(a'+b')a/a'$, and the concentration $Fa/10a'$ mg./100 ml., where F is the recovery factor obtained from data such as those of Table III.

TABLE II

RECOVERY OF BERCULON A FROM BLOOD (2 ML.) BY ESTIMATION AT λ_{max} 330 mμ
(Method II)

Added (μg.)	Found* (μg.)		Recovery (%)	
0	0.23	0.90	—	—
5	3.81	4.48	72	72
10	8.08	8.20	78	73
15	13.0	12.8	85	79
25	20.2	19.1	80	73
35	28.7	29.9	82	83
50	42.0	40.5	84	79
		Mean	78	
		Limit of error of mean (P = 0.05)	±3.1	

* Two separate experiments.

TABLE III

RECOVERY OF BERCULON A FROM MOUSE BLOOD (3 ML.) BY THE GRAPHICAL METHOD (Method III)

Added (μg.)	Found (μg.)	Recovery (%)
0	0	—
0.5	0.328	66
1.0	0.600	60
1.0	0.641	64
1.5	0.975	65
1.5	0.992	66
1.5	1.08	72
2.5	1.58	63
5.0	3.46	69
	Mean	66
	Limit of error of mean (P = 0.05)	±3.1

Notes.—The recovery of berculon A from blood by this procedure is given in Table III. Detailed work on the method shows that the low recovery is due to two factors: first, the incomplete (80 per cent) extraction of berculon A from blood (Tables I and II); second, an apparent loss (20 per cent) of berculon A due to the departure of the normal curve from linearity (Fig. 4). The normal curve is always concave with respect to the tangential straight line, and the increment *a* is therefore smaller than it would be were the curve approximately linear. It might be supposed at first sight that this concavity would introduce a constant error, and therefore that the recovery of different amounts would be affected disproportionately. This is not so, because the tangential straight line is shorter for smaller amounts of berculon A; it therefore cuts off a smaller part of the normal curve and the error due to the concavity is consequently less. Different amounts may therefore be estimated without serious error using a single recovery factor *F*. However, *F* should be established for blood of the species to be studied experimentally. The method is suitable for the estimation of from 0.5 to 5.0 μg. of berculon A. The curves shown in Fig. 4 illustrate the estimation of 3 μg. An amount of this order is found in 3 ml. of blood in therapeutic experiments. Less than 5.0 μg. of berculon A cannot be estimated directly by method II because such an amount absorbs no more strongly than an extract of normal blood, and the absorption of such an extract varies from animal to animal, and in the same animal at different times. For example, the absorption at 330 mμ of an extract of human blood increased by nearly 150 per cent half an hour after the subject had drunk half a pint of milk.

The method can be applied to berculon B, the recovery of which from blood is shown in Table IV. The whole of the apparent loss of berculon B, which is extracted quantitatively (Spinks, 1949), is due to the departure of the normal curve from linearity.

The application of the method to the measurement of low concentrations of berculon A and berculon B in dog blood is shown in Fig. 5. As in the mouse berculon B gave concentrations 4–5 times higher than those of berculon A.

A similar graphical technique was developed by Coggeshall and Glessner (1949) for the correction of background absorption in naphthalene estimation.

Photolability of thiosemicarbazones

All the methods described above must be carried out in dim light in order to prevent the rapid transformation of the thiosemicarbazones that occurs in strong light, including strong tungsten light. During early work on the development and

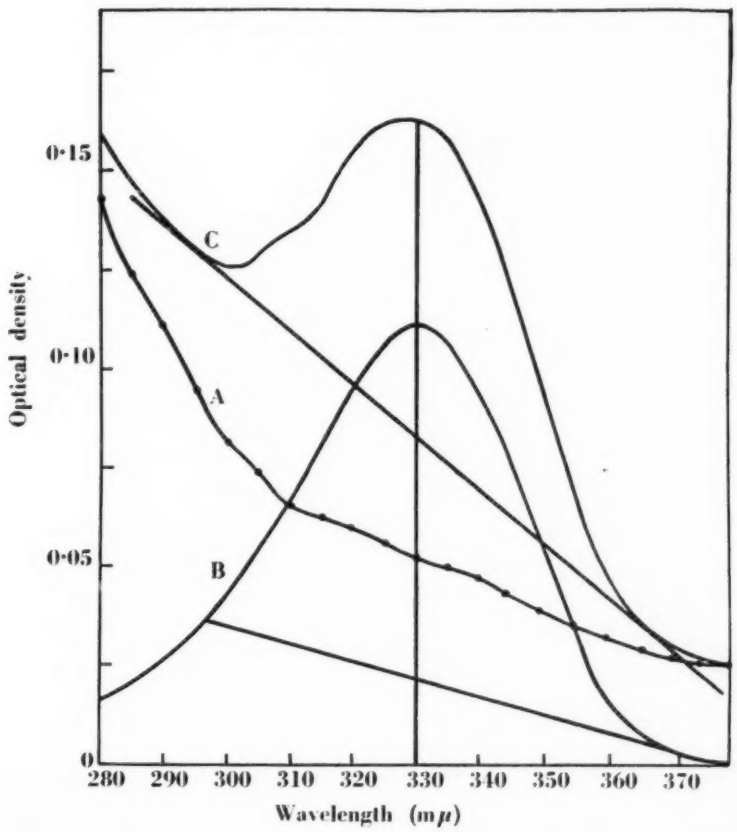


FIG. 4.—Estimation of berculon A by the graphical method: A, absorption spectrum of an extract of normal blood; B, absorption spectrum of an extract of 3 μg. berculon A; C, sum of A+B. For a full explanation, see text.

use of the methods described in a previous paper (Spinks, 1949) it was found that readings of standards remained normal and at about the values expected, although estimations were made in daylight. Later it was found that standards often gave low and erratic readings, and the results of several animal experiments had therefore to be abandoned. It was known that some thiosemicarbazones were photolabile; for example, *p*-dimethylaminobenzaldehyde thiosemicarbazone was slowly converted to a product with an entirely different absorption spectrum when irradiated in methanolic solution. The product was thought from its absorption spectrum to be a 1: 3: 4-thiadiazole formed by oxidative ring closure. Although a powerful ultra-violet lamp was used the transformation was very slow, and solutions of the compound in methanol did not appear to be labile in diffused daylight. Because of these findings no special precautions to avoid photodecomposition during estimations were taken until the erratic readings were observed. The problem was then re-examined and the difference between early and later findings was traced to three main factors. First, the photolability of the thiosemicarbazones is more marked in chloroform than in methanol, so that the work with methanol was misleading. Second, the early estimations were made in winter, at a time when fog

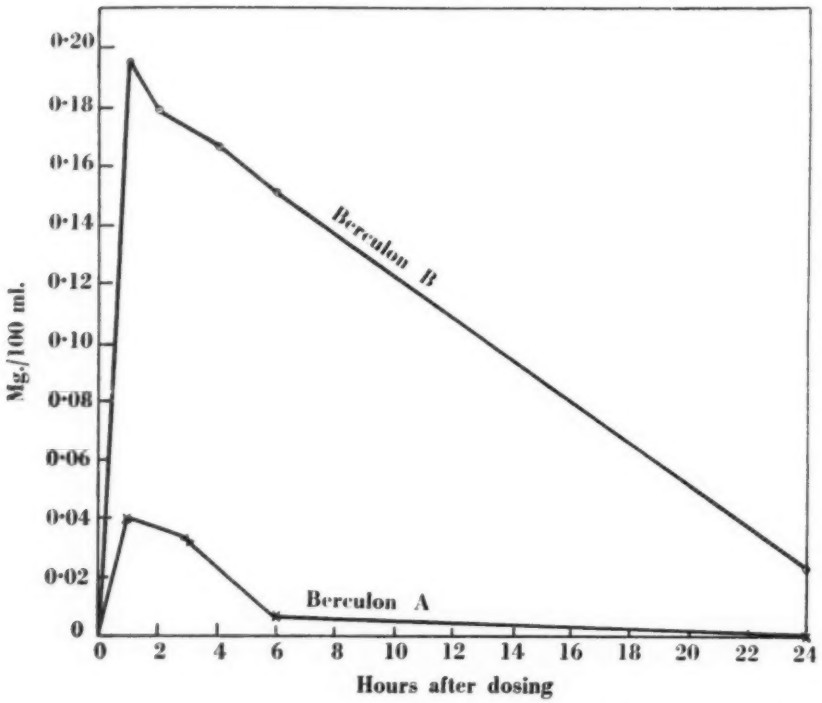


FIG. 5.—Blood concentrations of berculon A and berculon B in a dog after oral doses of 5 mg. per kg.

was very frequent. The later work was done during the exceptionally sunny weather of 1949. Third, the thiosemicarbazones undergo reversible photochange, the reversal occurring in the dark and therefore in the cell compartment of a spectrophotometer. A slight degree of photochange is consequently reversed before a reading can be taken. These findings were established qualitatively in daylight. Later quantitative

TABLE IV

RECOVERY OF BERCULON B FROM MOUSE BLOOD (3 ML.) BY THE GRAPHICAL METHOD (Method III)

Added (μg.)	Found (μg.)	Recovery (%)
0	0	—
0.5	0.459	92
0.5	0.444	89
0.5	0.428	86
1.0	0.735	73
1.0	0.722	72
1.5	1.09	73
1.5	1.28	85
2.5	2.14	86
5.0	3.94	79
5.0	4.28	86
	Mean	82
	Limit of error of mean (P = 0.05)	±5.1

work with ultra-violet lamps showed that the behaviour of the thiosemicarbazones was even more complicated than had been thought. The findings are illustrated by the following experiments with *p*-aminobenzaldehyde thiosemicarbazone (6198). They were the first quantitative ones carried out.

A solution of 0.5 mg. 6198 in 100 ml. chloroform was prepared in a dark-room under weak diffused tungsten light (40-watt lamp in an opal glass globe at a distance of five metres). In a 2-cm. cell it had an optical density of 1.82 at λ_{max} 342 mμ. The cell was placed 20 cm. from an Hanovia U.V. lamp and irradiated for accurately timed periods, the density at 342 mμ being rapidly read at the end of each period. The findings were as follows (Fig. 6): the optical density dropped rapidly to 0.94 after 7 minutes; after 10 minutes it was 1.00. This slight rise was thought to be due to an error of observation, since reading was made difficult by the reversal of the photochange which began as soon as the cell was placed in the cell compartment. Irradiation was therefore discontinued and readings were taken in the dark every 5 minutes. The optical density rose until it almost reached its first value. In order to see whether this double change could be repeated the same solution was again irradiated (Fig. 6); this time the optical density fell for 3 minutes only, and then only to 1.04; it then rose during *continued irradiation*, and had reached 1.22

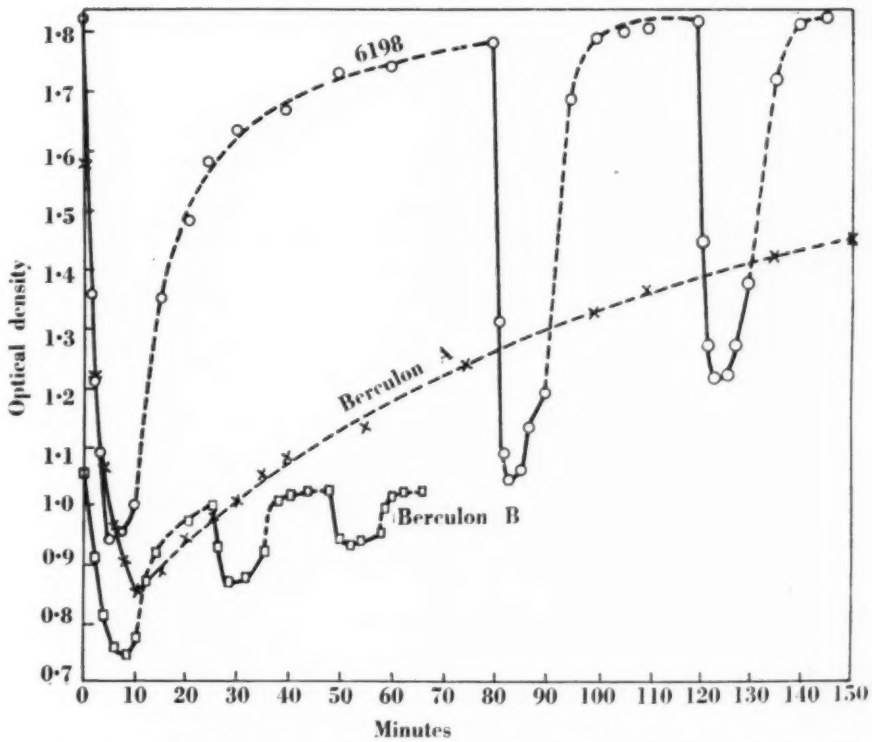


FIG. 6.—Change of optical density of chloroform solutions of 6198, berculon A, and berculon B during alternating periods of irradiation (continuous lines) and darkness (broken lines).

after 10 minutes. This rise continued during subsequent standing in the dark until the optical density was at its earliest value again. A third irradiation of the same solution confirmed the trend observed in the second. The minimum observed during this irradiation was higher than during the second, and the rise that occurred during irradiation was more marked (Fig. 6).

There was only one possible explanation of these results. The first photochange, which was reversible in the dark, was accompanied by a second photochange which was irreversible. The gradual accumulation of the product of this second change caused the trend shown by successive irradiations. Moreover, it was obvious that the reversible photochange was giving a product with a different absorption spectrum from the original compound, whereas the irreversible change was probably giving a product with a similar absorption spectrum. The last conclusion was confirmed by the following experiment. Another solution of 0.5 mg. 6198 in 100 ml. chloroform was prepared and the absorption spectrum was constructed. The solution was irradiated until no further change in optical density at λ_{max} 342 mμ occurred (Fig. 7). The optical density then remained constant during an

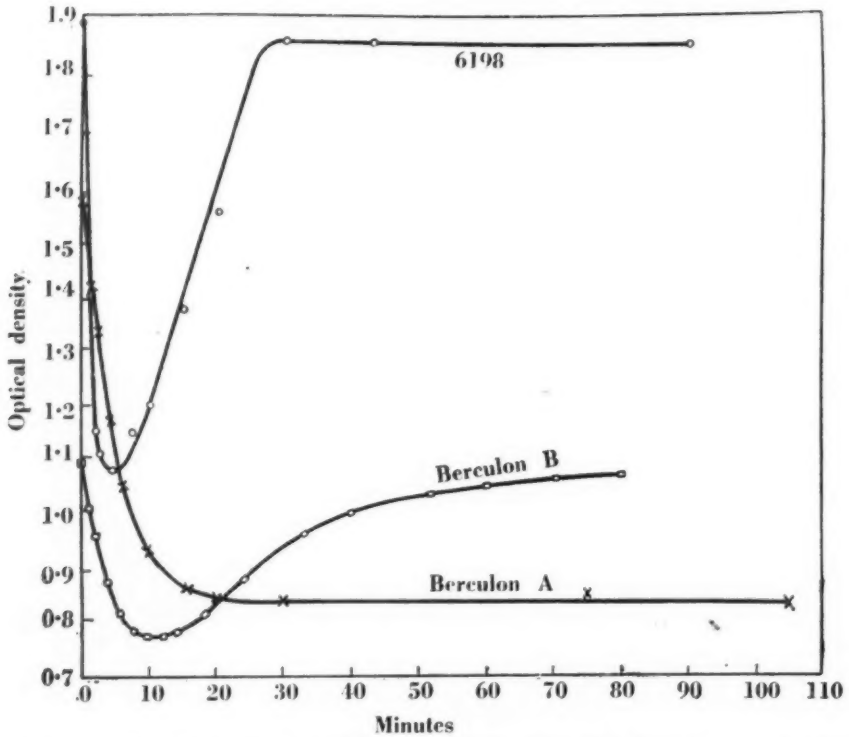


FIG. 7.—Change of optical density of chloroform solutions of 6198, berculon A, and berculon B during continued irradiation.

hour's irradiation and during an hour in the dark. The absorption spectrum was again constructed and was identical with the original one (Fig. 8).

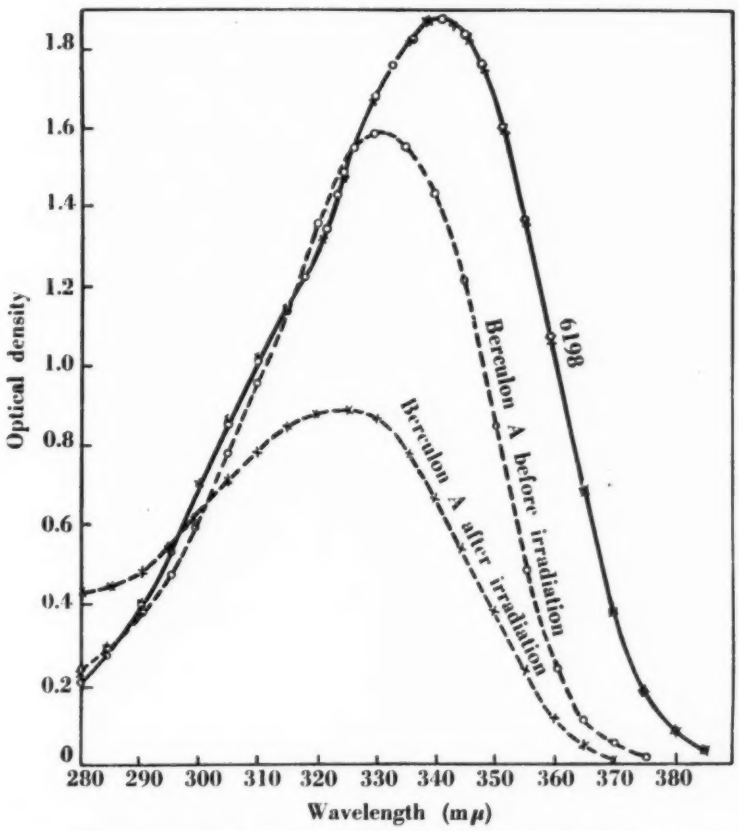
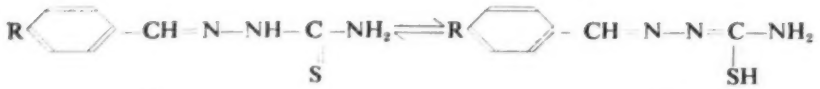


FIG. 8.—Absorption spectra of 6198 (continuous line) before (O) and after (×) irradiation; similar spectra of berculon A before and after irradiation until no further change occurred.

Before describing the transformations of berculon A it is convenient to summarize the behaviour of 6198 in the following diagram:

$$W \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} Y \longrightarrow Z$$

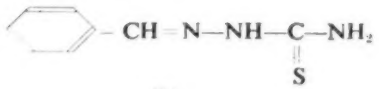
Compound *W* is the original one, *Y* is that formed initially in the light, *Z* is the product of continued irradiation. Compound *Z* may be formed from *W* instead of *Y*, although from the shape of the irradiation curve the route shown is the more likely. *W* and *Z* have identical spectra; that of *Y* is different. The reversibility of the *W*→*Y* transformation indicates that a simple tautomeric change is involved, and because a similar change is displayed by berculon B (see below) and *m*-methoxybenzaldehyde thiosemicarbazone (not described), this change must be independent of the nature and position of the substituent in the benzene nucleus, and must involve the thiosemicarbazone group. The obvious possibility is that shown:



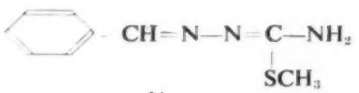
II III

These tautomers would be expected to have different absorption spectra. Because of the instability of *Y* in the 6198 series it was impossible to obtain its absorption spectrum or to suggest structures for *W* and *Y*. It was therefore fortunate that berculon A behaved differently from 6198. The nature of the difference is shown in Figs. 6 and 7. When berculon A was irradiated under the standard conditions used for 6198, the change, $W \rightleftharpoons Y$, occurred as with 6198, but no evidence of any formation of *Z* was obtained. Moreover, the dark reaction, $Y \rightarrow W$, was slow enough to allow an approximate absorption spectrum of *Y* to be obtained. This absorption spectrum is given in Fig. 8. It showed marked differences from that of the original berculon A, the maximal absorption being much reduced and at a slightly lower wavelength. Berculon B, on the other hand, behaved like 6198 (Figs. 6 and 7): a form *Z* was produced, and its spectrum was identical with that of the original berculon B (not shown).

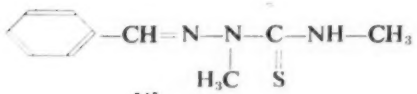
It remains to allot structures to forms *W* and *Y*, and to consider the possible nature of form *Z*. In order to do this it was necessary to obtain the absorption spectra of compounds possessing the unequivocal structures of tautomers II and III. The necessary compounds related to 6198, berculon A, or berculon B were not available, but those shown below (IV, V, VI) were. They were provided by



IV



V



VI

Dr. Hoggarth, to whom the author's thanks are extended. The absorption spectra are shown in Fig. 9. Those of benzaldehyde thiosemicarbazone (IV) and its N^2: N^4-dimethyl derivative (VI) were similar and indicated that the former exists preponderantly in the form shown as II above. The maximal absorption of the S-methyl derivative (V) was much lower, and was at a lower wavelength. The absorption spectra of forms *W* (original) and *Y* (initial irradiation product) of berculon A (Fig. 8) corresponded well with those of benzaldehyde thiosemicarbazone and its S-methyl derivative respectively, although both curves were shifted to longer wavelengths. It is therefore probable that the original (*W*) form of the thiosemicarbazones has the thiocarbonyl (C=S) structure, or is a mixture in which this structure predominates, and that the first irradiation (*Y*) product has the sulphydryl (C–SH) structure.

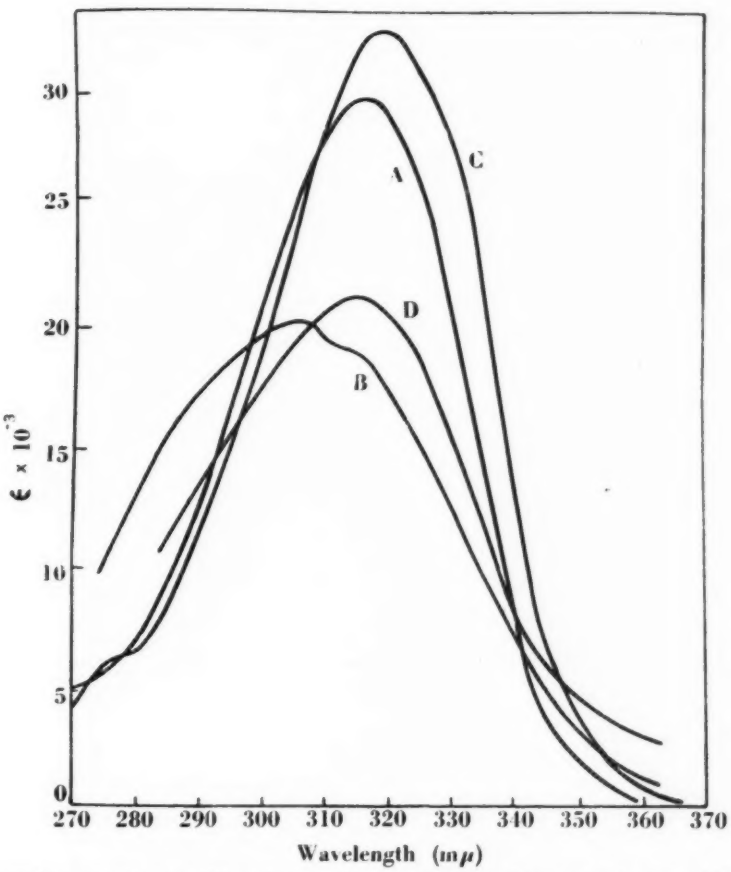
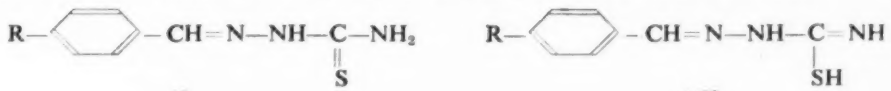


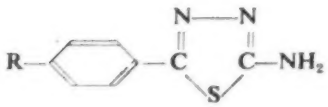
FIG. 9.—Absorption spectra, in chloroform, of benzaldehyde thiosemicarbazone (A), its S-methyl derivative (B), its N^2: N^4-dimethyl derivative (C), and its irradiation product (D).

This view was strengthened by examining the photolability of benzaldehyde thiosemicarbazone. It behaved almost exactly like berculon A, and an approximate spectrum of form *Y* was obtained (Fig. 9). When allowance is made for a partial return to form *W* during the construction of the spectrum, and for the unpredictable effect of the methyl group on the spectrum of the S-methyl derivative, it must be concluded that forms *W* and *Y* of benzaldehyde thiosemicarbazone also have the C=S and C–SH structures respectively. Further evidence is provided by the stability to irradiation of the S-methyl (V) and N^2: N^4-dimethyl (VI) derivatives of benzaldehyde thiosemicarbazone. In them the tautomeric change, II→III, is impossible, although in (VI) the other possible change, II→VII, could still occur.

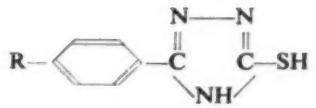


II VII

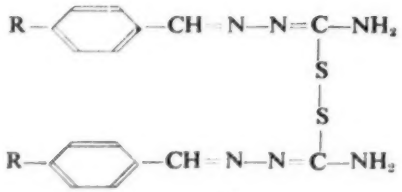
The nature of form *Z* has not been proved. The 1: 3: 4-thiadiazole (VIII) and 1: 2: 4-triazole (IX) which could be formed by oxidative ring closure are eliminated



VIII



IX



X

because they have very different spectra from the corresponding *W* form. The structure (X) would be expected to simulate (III) in spectrum, and so would the tautomer (VII). Another possibility is that *Z* is the initial form *W* stabilized in some way by continued irradiation. For example, a stabilizing substance might be formed from the solvent or from an impurity; or an impurity which stabilizes *Y* might be destroyed. Although at first sight this possibility does not seem very plausible it does explain the exact identity of the spectra of *W* and *Z*. If *W* and *Z* had different structures their spectra would be expected to differ at least slightly. It is also in accord with the behaviour of benzaldehyde thiosemicarbazone and berculon A. Their *Y* forms are more stable than those of 6198 and berculon B, in that they return only very slowly to *W* forms in the dark. Neither gave a *Z* form on continued irradiation. This is easily explained if *W* and *Z* are identical, because the return to *W* in the dark is then the same reaction as the production of *Z* on continued irradiation. Otherwise it is necessary to assume that two different reactions are equally affected by a single structural modification.

Since the isolation of the *Z* form of 6198 from chloroform would be difficult because of the low solubility of 6198, other solvents have been examined. It was hoped also that a solvent allowing analysis in daylight would be found. Fig. 10 shows the photolability of 6198 in methanol, butanol, ethylene dichloride, and tetrachlorethylene. The results should be compared with those in Fig. 6. Compound 6198 was fairly stable in methanol; it changed slowly in butanol, and rapidly in ethylene dichloride and tetrachlorethylene. In tetrachlorethylene it behaved like berculon A in chloroform, in that the *Y* form was stable to continued irradiation, and returned only slowly to the *W* form in the dark (not shown). In ethylene dichloride the *Y* form returned to *W* rapidly in the dark, as in chloroform. No formation of *Z* was observed in butanol, ethylene dichloride, or tetrachlorethylene. This finding provides some support for the theory that *Z* and *W* are identical, since it shows that *Z* is formed in a particular solvent only. A possible explanation (in part suggested by Mr. J. M. Thorp) is that the C–SH (*Y*) form is produced in light only in the presence of traces of water. The irradiation of chloroform would lead to the formation of phosgene ($CHCl_3 \rightarrow COCl_2 + HCl$) and dehydration of the solvent, with consequent stabilization of *W*. Yet another possibility (suggested by Dr. J. Madinaveitia) is that the HCl produced by irradiation forms a stable hydrochloride of *W*. Both these possibilities could readily be substantiated or

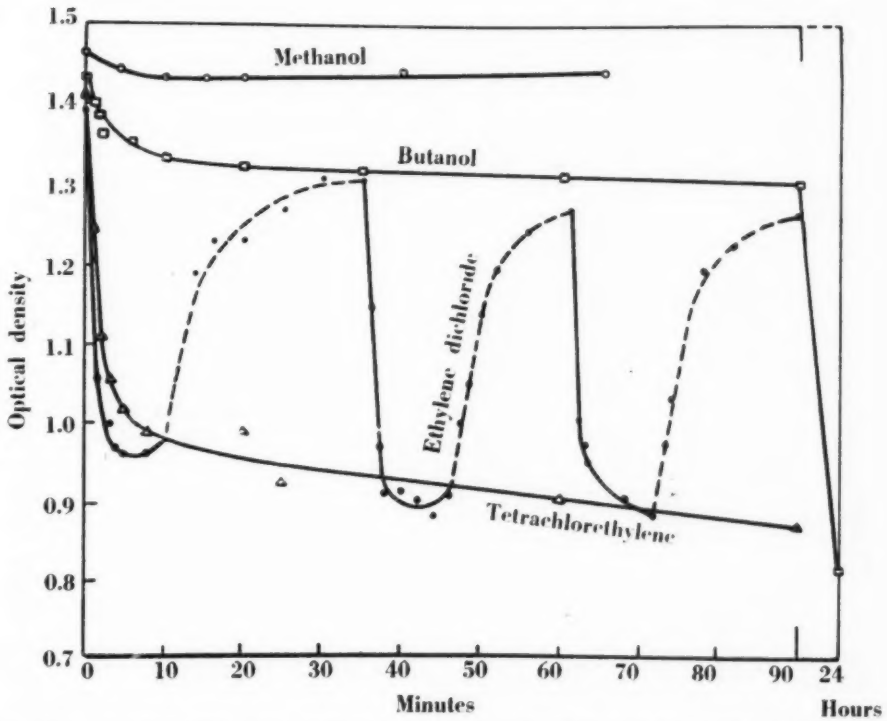


FIG. 10.—Change of optical density of solutions of 6198 in methanol, butanol, ethylene dichloride, and tetrachlorethylene during irradiation (continuous lines) or darkness (broken lines).

disproved by experiment. Unfortunately the author is at present unable to continue the study.

No solvent seemed likely to allow estimation of the thiosemicarbazones in daylight. Those of similar polarity to chloroform gave photolabile solutions of 6198; and methanol and butanol, in which 6198 was fairly stable, extracted much interfering material from blood.

SUMMARY

p-Aminobenzaldehyde thiosemicarbazone (6198) and *p*-acetylaminobenzaldehyde thiosemicarbazone (berculon A) have been estimated simultaneously by extracting them from blood with chloroform, reading the optical densities at 320 and 342 mμ, and solving simultaneous equations.

Compound 6198 is acetylated by the mouse to berculon A, but deacetylation of berculon A to 6198 has not been demonstrated in mouse, dog, or man. Berculon A can therefore be estimated directly in these species by reading optical density at λ_{max} 330 mμ only.

A graphical method of estimating berculon A or berculon B (8388; *p*-ethylsulphonylbenzaldehyde thiosemicarbazone) in blood in therapeutic concentrations of 0.02 to 0.2 mg./100 ml. is described. The direct technique cannot be used to estimate such small amounts because of the high and variable absorption of an

extract of normal blood. In the mouse and dog berculon A gives blood concentrations about a quarter as high as those of berculon B.

The photolability of the thiosemicarbazones is described. In chloroform the original form (*W*) of 6198 gives on irradiation a form *Y* which returns to *W* in the dark. Simultaneously there is produced a form *Z* which is stable in light and dark. The spectra of forms *W* and *Z* are identical, and suggest that *W* at least has the structure —CH=N—NH—C(=S)—NH_2. The *Y* forms of benzaldehyde thiosemicarbazone and berculon A return only slowly to *W* in the dark, and their spectra suggest that they have the structure —CH=N—N=C(–SH)—NH_2. The structure of form *Z* has not been proved, but it may be identical with *W*, stabilized in some way by continued irradiation. A possible manner of stabilization is discussed.

Because of their photolability thiosemicarbazones must be estimated in the dark-room. Weak, unfiltered tungsten light may be used.

The author is greatly indebted to Dr. A. R. Martin, Dr. E. Hoggarth, and Mr. J. M. Thorp for helpful discussion and to Mrs. E. Powell and Mr. R. Hughes for technical assistance.

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UNMASKING, AFTER CHOLINERGIC PARALYSIS BY BOTULINUM TOXIN, OF A REVERSED ACTION OF NICOTINE ON THE MAMMALIAN INTESTINE, REVEALING THE PROBABLE PRESENCE OF LOCAL INHIBITORY GANGLION CELLS IN THE ENTERIC PLEXUSES

BY

N. AMBACHE

*From the Medical Research Council Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C.*1

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There are several indications that the motor nerves to the intestine are susceptible to the paralysing action of botulinum toxin. Dickson and Shevky (1923) noticed a distinct interference with the intestino-motor function of the vagus in their experiments on cats; and most accounts refer to the obstinate constipation which is a prominent feature of human botulism.

The present experiments, conducted on mice and rabbits, aim at demonstrating this condition in isolated intestinal preparations. In mice systemic botulism was produced, and peristaltic activity was studied in surviving portions of the intestine. In rabbits the toxin was injected locally into the wall of an intestinal segment, which was examined later after the death of the animal from a spread of intoxication. Further information was gained by studying the response of these poisoned segments to nicotine; with this drug it is possible to stimulate the same ganglion cells in the intestine as are involved in the mediation of peristaltic reflex activity, and the extinction of the motor response to nicotine can therefore be used equally well as an index of the paralysis in the myenteric plexus.

Botulinum toxin has a selective affinity for cholinergic nerve endings, and produces effects resembling denervation (Guyton and MacDonald, 1947; Ambache, 1949, 1951). It is generally assumed that the nerve fibres originating from the cells of the myenteric plexus are cholinergic. These motor neurones should therefore also succumb to the paralytic action of this toxin, and should cease to respond to nicotine-stimulation of their ganglion cells. It has been found that, when the action of nicotine on these motor neurones is paralysed by botulinum toxin, an opposite action of nicotine is unmasked at the same time, revealing a stimulation of ganglion cells which are *inhibitory*, and appear to be the cell bodies of short adrenergic neurones.

METHODS

Botulinum toxin.—For the experiments on mice, which were performed in 1949 in Guy's Hospital Medical School, a type A toxin broth was used. It was kindly supplied by Dr.

C. R. Amies, then at the Lister Institute. It contained 200,000 minimum lethal doses per ml. For the later experiments on rabbits two powder preparations of toxin A were available. The first preparation, toxin A_1, was identical with that used two years ago (Ambache, 1949); its LD50 by intravenous injection was then 0.1 μg./kg. of mouse. The second toxin, A_2, was from a more recent batch, available to me through the kindness of Drs. D. W. Henderson and J. Keppie, of the Microbiological Research Station, Porton. This batch had the further advantage of greater purity. Since its toxicity (LD50 by intraperitoneal injection, 0.035 μg./kg. mouse) was three times as great as that of A_1, the dosage was scaled down accordingly. Both powders were taken up in sterile saline (0.9 g. NaCl per 100 ml.), but only some of the toxin went into solution, the rest remaining in suspension. After breaking up the larger visible particles in this suspension, the toxin was injected with the minimum delay; freshly made toxin solutions were used for each experiment on rabbits.

Experiments on mice (*systemic botulism*).—Four mice were injected subcutaneously, or intraperitoneally, with suitable dilutions (in unbuffered saline) of the culture-broth. These dilutions lost their toxicity in 3–4 weeks, presumably owing to contamination with alkali from the glass container; in the absence of a buffer, the resulting change in *p*H would naturally lead to destruction of the toxin. For this reason some of the mice had to be reinjected with a fresh dilution of toxin. The animals were either allowed to die of the intoxication or had to be killed before its full development. Immediately after death a portion of the small intestine (ileum) was excised, and was examined for peristaltic activity in a 5-ml. organ bath. A liquid bolus of Tyrode solution was entrapped in the piece of intestine, which was ligated at both ends; the bolus was intended to act as a distending stimulus, which in the normal intestine produces peristalsis (Ambache, 1946).

Experiments on rabbits (*local injections of toxin into the intestinal wall*).—The rabbits were of mixed stock and weighed 1.4 to 3.35 kg. The intestinal injection was performed under ether anaesthesia, with the usual precautions. The abdomen was shaved and the gut delivered on to a sterile towel through a long midline incision. The period of exposure of the viscera during the injection was kept as brief as possible (*ca.* 5–10 min.).

FIG. 1.—Method of local injection of toxin into intestinal wall. The whitish appearance round the syringe needle (at A) is due to a thin sheet of air injected in advance of the toxin and spreading between the longitudinal muscle-layer and its peritoneal covering. Small air-bubbles have spread along the gut and can be seen as far as B. Subsequently the intestine is similarly injected, first with air and then with toxin, on its reverse side, from the other end of the segment (i.e., at B) with the needle pointing towards A (for details see text).

The aim was to place the toxin subperitoneally, i.e., in between the longitudinal muscle-layer and its peritoneal investment. This was done by creating an artificial separation between these two layers, preparatory to the injection of toxin, by injecting air as follows. The syringe was loaded with the appropriate volume of the toxin-solution and 0.3–0.5 ml. of air also drawn into it; thus, from the same syringe, air and toxin could be injected into the gut in succession, the air as the forerunner of the toxin. When the preliminary injection was in the desired anatomical plane, air could be seen spreading out, sometimes for a considerable distance along the intestine, in several rows of bubbles, or as a sheet of air producing the whitish appearance shown at A in Fig. 1. This appearance was always taken as a criterion of success before proceeding with the introduction of toxin, which was then observed entering the artificial space so created and displacing the bubbles of air.

At first toxin was injected at one end of the intestine, but a more satisfactory procedure was evolved for the last thirteen experiments; this consisted of injecting air and toxin at two points, 5–10 cm. apart, in such a way as to produce a high concentration of toxin in the intervening stretch of intestine, which was later used for examination. The dose of toxin was divided into equal halves. The first half was injected in a caudal direction at a point marked A in Fig. 1, on one side of the gut, at about 42–130 cm. from the pylorus. The intestine was then turned over, and the remainder of the toxin was injected at a point (marked B in Fig. 1) on its under side, 5–10 cm. below A, with the needle pointing, this time, in an oral direction. This left a segment of gut AB, injected with toxin on both sides, marked by two small haemorrhagic puncta at A and B, and easily identifiable after death. The distances from the pylorus were measured *post mortem* in nearly all the experiments, as well as, sometimes, the distance from B to the ileo-caecal valve.

After the injection the intestines were replaced and the abdominal wall was sewn up in two layers, the muscular layer with an autoclaved cotton-thread and the skin with catgut. Recovery from the anaesthetic was prompt.

The total amounts of toxin injected into an intestinal segment were: of toxin A_1, 1–2.2 mg. when one end was injected (4 experiments), and 8 mg. when both ends were injected (1 experiment); of toxin A_2, 0.4–0.8 mg. when one end was injected (2 experiments), and 0.35–2 mg. when both ends were injected (8 experiments).

The rabbits died later on the same day, in $3\frac{2}{3}$–$7\frac{1}{2}$ hr. An indication of the impending death of the animal was obtained from its pulse rate, which fell progressively after a time and to very low values just before death. The phenomenon was recorded electrocardiographically, but its nature has not been further investigated. One rabbit was killed two hours after the injection when showing signs of intoxication.

The moment the animals were dead the abdomen was reopened and the appropriate piece of gut (usually of 4 to 5 cm., sometimes of 6 to 7.5 cm. length) was excised between ligatures. The preparation was then tied at one end on to a stainless steel-wire strut and transferred at once to the warm, oxygenated, Tyrode's solution in the organ bath (approximately 10 ml.), into which the strut fitted tightly. The preparations were made from the middle of the injected segment so as to exclude the traumatized, slightly haemorrhagic region in the vicinity of the puncta. Intestinal movements and responses to drugs were recorded with a gimbal lever writing sideways.

The Tyrode's solution was made without magnesium and with 0.11 g. $NaHCO_3$ per 100 ml. It was aerated with a gas mixture of 95 per cent O_2 and 5 per cent CO_2, which was calculated to produce a *p*H of 7.2 in the bath. For some experiments the Tyrode was made in glass-distilled water, for others in copper-distilled water; there was no difference in the results.

Drugs were usually left in the bath for a period of 30 sec. (timed by a stopwatch) unless otherwise stated.

Two experiments (Figs. 4 and 10) were performed at the Instituto Biologico, São Paulo, Brazil, in the department of Biochemistry and Pharmacodynamics, through the courtesy of Dr. M. Rocha e Silva. In these experiments the poisoned intestinal segment was suspended, with its lumen *open*, in a 7-ml. bath, and its movements were recorded with a compensated, linear, frontal-writing lever (Schild, 1946). The Tyrode solution used was made with glass-distilled water and contained magnesium; it was oxygenated with air.

Control experiments were performed not only on intestinal preparations from a normal rabbit killed by concussion but also on four preparations previously injected (under ether) with " boiled toxin " (0.35–1 mg. of toxin A_2). The toxin solution was sealed in ampoules which were heated in boiling water for 45–90 min.; the ampoules were shaken from time to time during the heating in order to re-disperse the coagulum. The injection technique was exactly as described above. These rabbits recovered rapidly from the operation, and were not displaying any abnormality when they had to be killed by concussion 6½ to 23 hours later.

In addition, a preparation was examined from a rabbit which died 1 hr. 35 min. after an intravenous injection of 4 mg. of A_1 (dissolved in a 50 per cent (v/v) glycerinated buffer).

Results

Loss of peristalsis in mice

Mice were chosen for these experiments because of the ease with which peristaltic contractions can be demonstrated *in vitro*, in normal preparations from these animals, if the precaution is taken of filling the lumen with a fluid which acts as a " liquid bolus " and provides a suitable stimulus for peristaltic activity.

Of the four mice injected with suitable dilutions of the toxic broth, only two (Nos. 3 and 4 in Table I) exhibited severe terminal symptoms of a generalized locomotor paralysis. No. 1 died, and No. 2 was killed, before the paralysis in the limbs was fully developed; the presence of peristaltic activity in these two animals (weak in No. 2), and of a response to 25 and 50 μg. of nicotine, was therefore consistent with their general condition. Their inclusion in Table I is intended both as a control and to show that it is possible for various organs to escape complete nervous paralysis in systemic botulism.

In mice Nos. 3 and 4, which were severely paralysed, there was no peristaltic activity. In the absence, or presence, of eserine the response to the customary doses of nicotine was either minute or absent, but the gut still contracted powerfully in response to acetylcholine (0.1–1 μg.).

Experiments on rabbit's small intestine

The response to nicotine.—The addition of nicotine to the bath for 30 seconds, in doses ranging between 5 and 300 μg., produces a contraction of the rabbit's intestine; after the nicotine has been washed out the preparation relaxes again. The effect of a given dose is repeatable at 3 to 5 minute intervals and the preparation remains responsive to the stimulating action of nicotine for 2½–5 hours; it may even become more sensitive to nicotine in the course of an experiment. In some instances, after the nicotine has been washed out, relaxation proceeds below the original base line, and this state of relaxation persists for several minutes despite frequent washing. During this period the amplitude of pendulum movements is often reduced; an example of this is given in Fig. 2. Since this post-nicotine phase of inhibition was never seen in the absence of a preceding nicotine contraction,

TABLE I

LOSS OF PERISTALSIS AND OF THE RESPONSE TO NICOTINE IN MICE POISONED WITH BOTULINUM TOXIN (BROTH)

MLD = Minimum lethal dose

Mouse No.	Dose of toxin (subcutaneous except in expt. 1); and age of toxin dilution	Terminal symptoms and progress	Peristalsis	Response to nicotine
1	200 MLD (fresh) intraperitoneally	Immobile at 17 hr., but able to hang on with hind limbs. *Died* 18 *hr.*	Present	Responds to 50 μg.
2	10 MLD (fresh)	Slow sliding gait. Able to grasp with its hind limbs. *Killed* 29½ *hr.*	Present (but weak and infrequent)	Responds to 25 and 50 μg.
3	5 MLD (fresh)	Advanced locomotor paralysis in all four limbs. Unable to support its weight. When lifted, performs abortive movements of hind limbs. Dribbling urine. *Died* 50 *hr.*	None	1. Minute response at first to 25 and 50 μg. 2. 30 min. later, no response to 50 μg. in the presence of 1 μg. eserine
4	5 MLD (toxin 7 days old)	Unable to right itself or support its weight. Weak abortive movements when lifted. Lachrymating. *Died* 102 *hr.*	None	1. No response to 5, 15, and 75 μg. 2. Small response to 100 μg. in the presence of eserine. Followed by a small response to 75 μg.

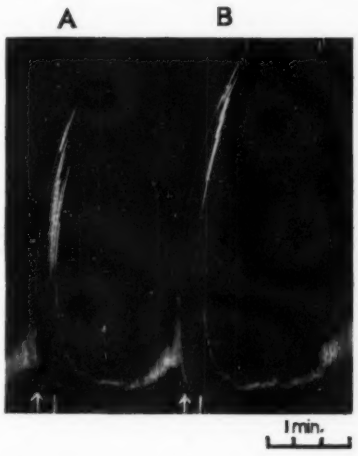

FIG. 2.—Rabbit's ileum (63 cm. below pylorus) of animal killed 6½ hours after local injection of 0.35 mg. inactivated toxin A_1. Contractions to 100 μg. (at A) and 200 μg. (at B) of nicotine. Note the after-effect, i.e., relaxation and reduced pendular movements.

i.e., in the poisoned preparations (see below), its development seems to depend on whether the nicotine has induced a contraction or not. In this it bears a resemblance to a phenomenon of depression, observed by Cantoni and Eastman (1946), subsequent to powerful contractions elicited by other drugs.

The same kind of response to nicotine as on the normal intestine was obtained (*a*) on four preparations subjected to previous local injections of " boiled toxin "; the response of one of these is shown in Fig. 2; (*b*) on the duodenum of an animal injected with active toxin at the other extremity of the gut (Fig. 3); and (*c*) in a

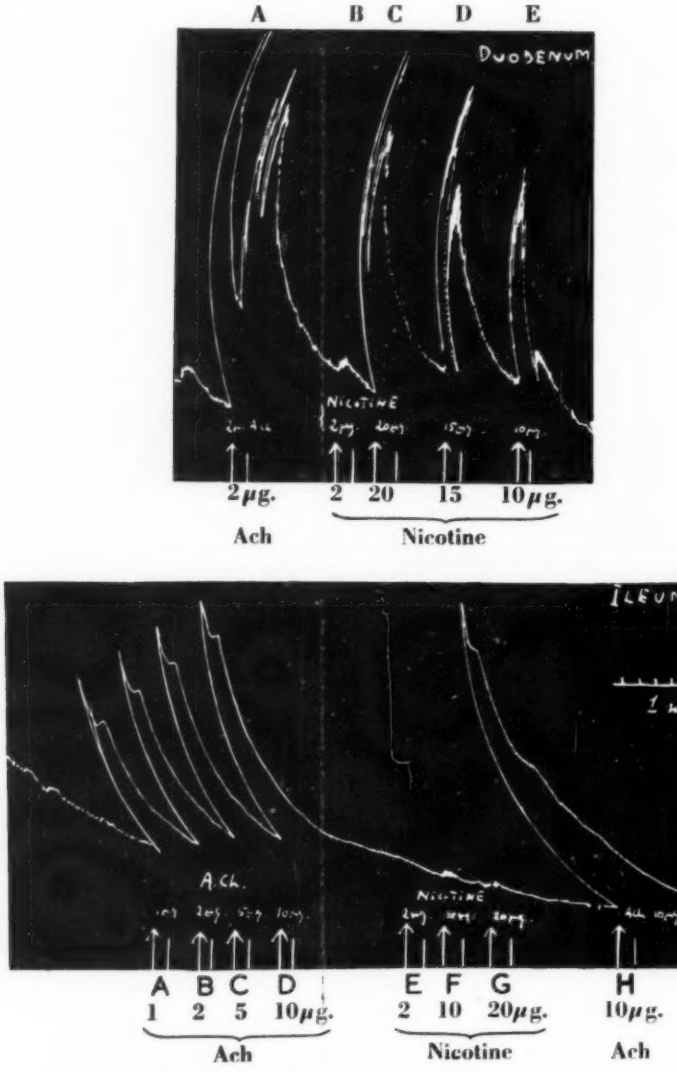

FIG. 3. — Duodenum (upper tracing) and lower ileum (bottom tracing) of rabbit (2.2 kg.). Death 5 hours after injection of 1 mg. botulinum toxin A_1 into ileal segment, from which lower tracing was obtained. Both preparations responded to acetylcholine, but only the duodenum to nicotine. Acetylcholine in upper tracing at A, in lower tracing at A, B, C, D, H. Nicotine in upper tracing at B, C, D, E, and in lower tracing at E, F, G.

preparation from an animal which died after an intravenous injection of a large dose of toxin.

An initial relaxation, instead of the usual contraction, was only seen once after the addition of nicotine to the bath; this was in one of the preparations which had been subjected to previous local injection of " boiled toxin." It occurred when the preparation had been in the bath for $4\frac{1}{4}$ hours. During the first $1\frac{1}{2}$ hours nicotine elicited only motor responses. Thereafter the preparation was left at rest

for about 1½ hours, and then the first administration of 5 μg. nicotine caused pure relaxation, but this effect could not be repeated with subsequent doses of nicotine. This exceptional relaxation was of the same kind as that described later for poisoned pieces of intestine.

The experiment in which toxin was injected into the wall of the intestine, at some distance away from the piece subsequently used for pharmacological examination, showed that the injection of toxin into one end of the gut may leave the other end unparalysed. Direct spread of toxin over such a distance, which is more than 2 m., was not to be expected. But, in addition, the experiment showed that, after systemic absorption of what proved to be a lethal amount of toxin, the local concentration of toxin in the gut at a distance from the site of injection was inadequate to abolish the motor response to nicotine, i.e., to produce paralysis of cholinergic nerves. This was also true in the other control experiment in which the animal was killed by a large dose of toxin injected intravenously. Concurrent observations on the pupil, in both experiments, showed that the paralysis of the sphincter pupillae was also incomplete in these animals. When the pupillary reaction to light was taken as another index of cholinergic paralysis, it was found in several experiments that, even just before death, the iris would still respond to light, though at times sluggishly.

These results showed the necessity of achieving a high local concentration of toxin in an organ, if complete paralysis of its cholinergic nerve supply was desired.

The response to nicotine after local injection of active toxin.—When a segment locally injected with active toxin was suspended in the organ bath after the death of the animal from general intoxication, a difference in the response to nicotine from the normal was observed either straightaway or during the further course of the experiment. A total of fifteen botulinized preparations from different animals was examined. In all of them there was a loss of the normal intestino-motor action of nicotine (dose : 5–300 μg.). In eight this extinction of the motor response to nicotine was present *ab initio*; in six others weak contractions to nicotine, and in a seventh a slight augmentation of pendulum movements, were present at first but were soon lost. In twelve out of these fifteen experiments nicotine produced inhibitory responses, in six of them *ab initio*.

Two preparations demonstrated strikingly the change-over in the nicotine response which is produced by the toxin, because this reversal phenomenon occurred *post mortem*, some time after suspension of the poisoned intestinal segment. For instance, in the experiment of Fig. 4, 50 and 100 μg. of nicotine could still elicit powerful contractions from the poisoned gut 62–67 minutes after death; but after 147 minutes this was no longer possible. Both doses now produced relaxation (Fig. 4, C and D); and the same was true at 196 minutes (F). The rapidity with which this reversal in the action of nicotine may occur is shown in another experiment in which the rabbit had died three and a half hours after the local injection of 8 mg. of toxin A_1. Nineteen minutes after death 100 μg. of nicotine was still motor; but five minutes later 50 μg. produced relaxation, and after another hour a 100–μg. dose was inhibitory as well. In yet a third experiment of a slightly different type the rabbit was *killed* during the development of generalized symptoms, two hours after the local injection of 2 mg. of toxin A_2. The first response to nicotine of the

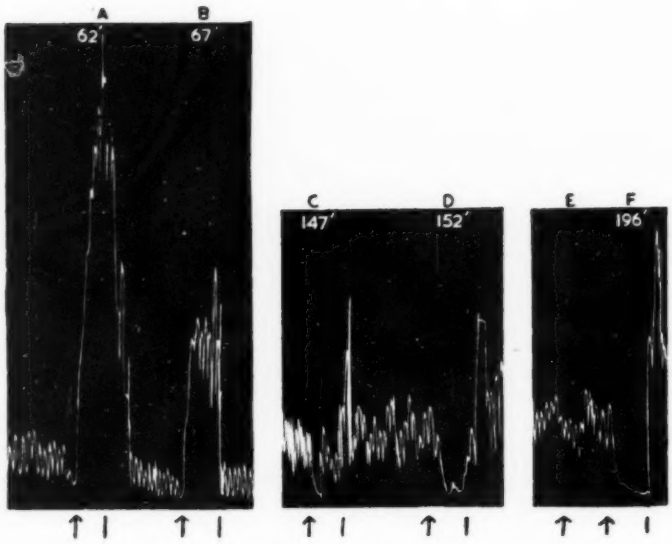

FIG. 4.—Ileum of rabbit (1.5 kg.) previously injected at both ends with 0.8 mg. botulinum toxin A_2, showing the change-over from a motor to an inhibitory response to nicotine. Death of animal 4½ hours after injection. The minutes on top of tracing refer to time after death. B and C, 50 μg., A, D, and F, 100 μg. nicotine; at E, 10 μg. benadryl.

suspended segment, when tested thirty minutes after death, i.e., only two and a half hours from the time when the toxin was injected, was already inhibitory.

Details of the twelve experiments in which nicotine produced the inhibitory response are as follows. In the first six experiments, four with toxin A_1 and two with toxin A_2, the portion of intestine destined for later use was injected on one side only and just at one end. In all there was a loss of the motor response to nicotine, either immediately or soon after suspension. It was in the course of these experiments that the appearance of distinct inhibitory responses to nicotine

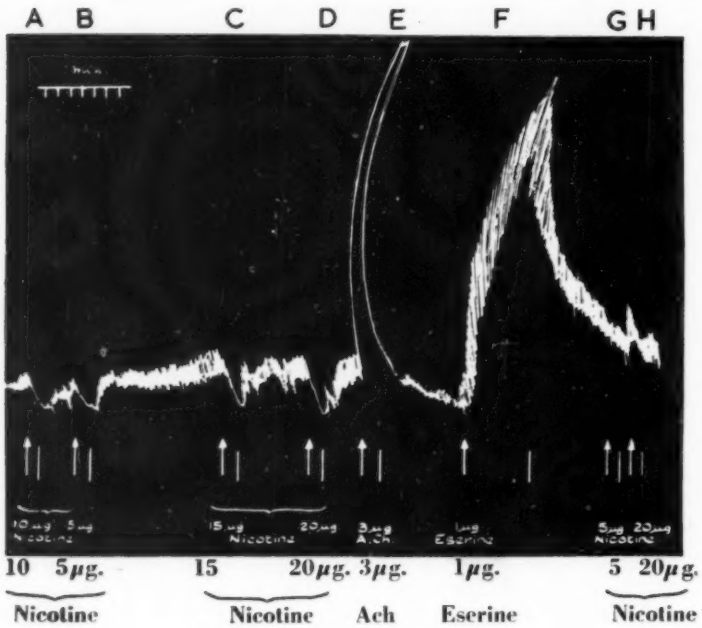

FIG. 5.—Jejunum of rabbit (3 kg.) with 2.2 mg. of botulinum toxin A_1. Injection at one end only of intestinal segment. Death after 6¾ hours. Tracing begins 65 minutes after death. Effects of nicotine (A, B, C, D, G, and H). acetylcholine (E), and eserine (F) (for details see text).

was first noted; one of them is illustrated in Fig. 5. Even when the motor response to nicotine was lost, the otherwise normal behaviour of the intestine was indicated by the presence of pendulum movements and of strong motor responses to acetylcholine and to eserine (see Figs. 3 and 5); but after eserine there was no outbreak of large peristaltic contractions of the type seen in normal preparations (Ambache, 1946).

In the experiment of Fig. 3, the loss of motor response to nicotine in a segment of ileum injected with toxin is contrasted with the normal response of a duodenal segment about 2 m. away from the site of injection.

In the last nine experiments (one with toxin A_1 and eight with toxin A_2) toxin was injected at both ends and on reverse sides of the chosen segment of intestine. This improvement in technique, combined with the institution of a higher dosage of the A_2 toxin, appeared to achieve a complete cholinergic paralysis with greater regularity. In four out of the nine experiments contractions could not once be evoked by nicotine, even early in the experiment; instead, inhibitions were recorded. The response to the whole gamut of nicotine doses (5–300 μg.) in this normally stimulating range was tested in three of these four experiments, and was inhibitory from start to finish; such an experiment is illustrated in Fig. 6.

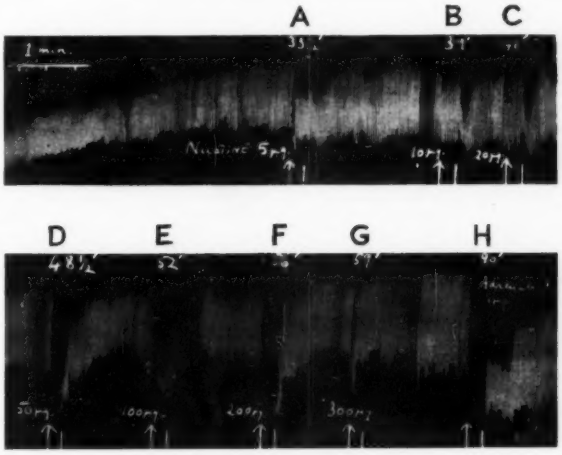

FIG. 6.—Small intestine of rabbit (2 kg.), 85 cm. below pylorus, injected with 0.8 mg. botulinum toxin A_2. Death after 5 hours. Minutes on top of tracing refer to time after death. A to G, effects of increasing doses (5, 10, 20, 50, 100, 200, and 300 μg. respectively) of nicotine; H, 1 μg. adrenaline (for details see text).

When higher doses of nicotine (2 mg.) were tested, traces of a motor action could be detected only once in three experiments; this was the brief contraction seen in Fig. 7 at C. Even this remnant of a motor action was lost when the same dose was tested again 3–4 hours later, when it was replaced by a distinct relaxation (at I) which in the other experiments was seen from the start.

The inhibitory reaction to nicotine in the poisoned intestinal segments has to be distinguished from the Cantoni and Eastman effect mentioned earlier (page 55). The latter is seen only *after* a large stimulating dose of nicotine is washed out of the organ bath. It lasts for several minutes and appears to be consequential upon the contraction preceding it; it is also seen after contractions produced by other drugs, e.g., by acetylcholine, and may originate in a temporary exhaustion of some energetic metabolite in the gut (Cantoni and Eastman, 1946).

On the other hand, the inhibitory phenomenon observed in the botulinized gut occurred regularly in the absence of any preceding contraction. It could be

elicited by small doses of nicotine (5–20 μg.); it developed at once when the nicotine was introduced into the bath and began to vanish as soon as the bath fluid was renewed. In no instance was a prolonged refractory state observed thereafter, whatever the previous dose of nicotine.

Analysis of the inhibitory action of nicotine on botulinized intestines

The inhibitory action of nicotine on the botulinized gut could be shown to be due to stimulation of nerve cells in the wall of the intestine, because it no longer occurred when these were paralysed either by large doses of nicotine or by hexamethonium.

Large doses of nicotine.—It was found that when the dose of nicotine was stepped up in a sequence, from 100–200 μg. to 300 μg. or 2 mg., the resulting inhibition decreased both in magnitude and duration, instead of showing further deepening (Figs. 6, G, and 7, I). This phenomenon may be taken to indicate the onset of ganglion-cell paralysis at the higher dose-level.

In untreated normal intestine it is easy to show that large doses of nicotine leave behind them a state of paralysis of the ganglion cells; when the intestine is in that condition small stimulating doses of nicotine are no longer able to contract it. This phenomenon of paralysis is known to persist for some time after a large dose of nicotine is washed out. If the inhibitory action of nicotine on the poisoned gut were also due to stimulation of ganglion cells, it too should be abolished after a paralysing dose of nicotine and then show gradual recovery in the course of time. Such an effect could, in fact, be observed in several experiments and an instance thereof is illustrated in Fig. 7.

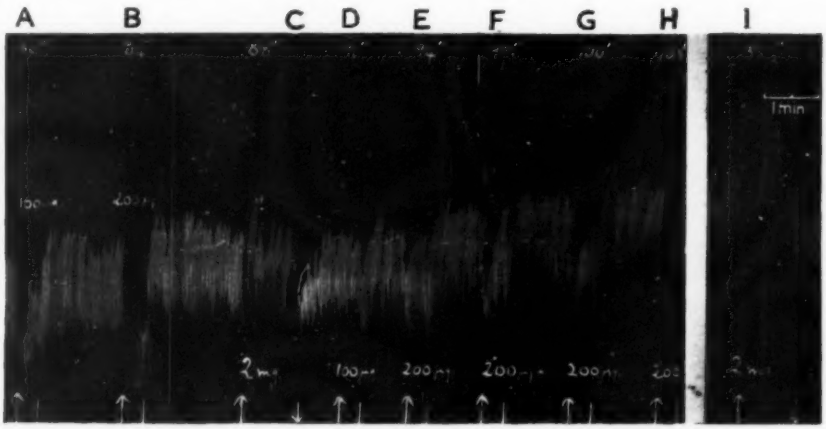

FIG. 7.—Small intestine of rabbit (2.6 kg.), 90 cm. below pylorus, injected with 0.75 mg. botulinum toxin A_2. Injections at both ends of the segment. Death in 5½ hours. The minutes on top of the tracing refer to time after death. Abolition of inhibitory effect of small doses of nicotine (100 μg. at A and D; 200 μg. at B, E, F, G, and H) by a large dose, 2 mg. at C. At I, a further 2 mg. nicotine (for details see text).

Against this interpretation of a nicotine-paralysis of " inhibitory ganglion cells " the argument may be put forward that after large doses of nicotine the sensitivity of normal preparations is reduced not only to ganglion-stimulating substances but

to others as well, and that in this condition even tone and spontaneous activity are diminished. Therefore the removal of the inhibitory effect of small doses of nicotine by large doses need not necessarily be a sign of ganglion paralysis. This argument does not invalidate the interpretation given above. It is true that, on the untreated preparation, paralysing doses of nicotine leave in their wake a condition of reduced responsiveness, but these doses of nicotine first produce contraction in the normal intestine, and strong contractions are generally followed by a period of reduced responsiveness of the intestine, a condition which has been referred to as the Cantoni-Eastman effect. In the poisoned preparation, however, nicotine has lost its motor action, and consequently the after-effect, whereby rhythmic activity and tone are depressed after large doses of nicotine, is absent; nevertheless, the inhibitory action of small doses is abolished. The deduction that this result is the outcome of ganglion paralysis is borne out by the experiments in which hexamethonium was used to paralyse the ganglia instead of large doses of nicotine.

The fact that there was no " depression " of spontaneous movement and tone after large doses of nicotine in botulinized preparations is interesting in another context. Since nicotine *per se* does not appear to exert a depressant action on the muscle fibres in the poisoned gut, it seems unlikely that it does so in normal preparations, where the possibility of such a depressant action on the fibres themselves may have been envisaged in the light of Bernheim's (1933) work, and was not clearly excluded more recently by Feldberg (1950a, b).

Hexamethonium.—The strong ganglion-blocking action of hexamethonium was discovered by Paton and Zaimis (1949). Its ability to abolish reversibly the motor effect of small doses of nicotine on the normal intestine has recently been described by Feldberg (1950b). The experiment of Fig. 8 shows that it can also abolish the other, inhibitory, action of nicotine which is seen on the poisoned preparation. This effect of hexamethonium is also reversible and can be obtained repeatedly on

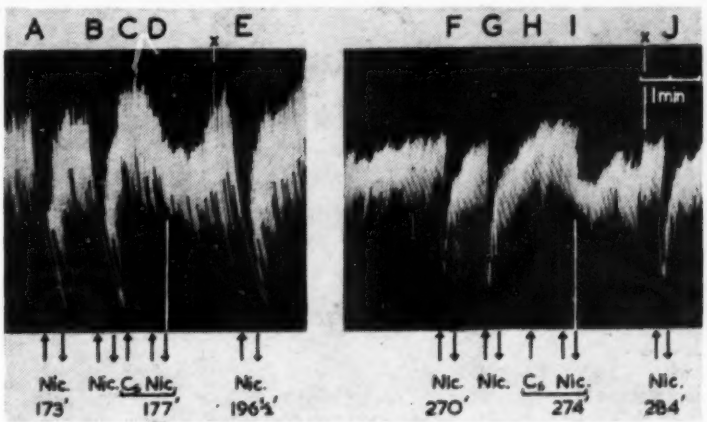

FIG. 8.—Small intestine of rabbit (2.5 kg.), 61 cm. below pylorus, injected with 2 mg. of botulinum toxin A_2. Injections at both ends of intestinal segment. Killed after 2 hours. The minutes at the bottom of the tracing refer to time after death. Abolition of inhibitory response to 100 μg. nicotine (at A, B, D, E, F, G, I, and J), by 2 mg. hexamethonium iodide (C6) (at C and H). Nicotine given at D and I in the presence of C6; both washed out together at the white line. At × drum stopped for several minutes (for details see text).

the same preparation. In the experiment of Fig. 8, the preparation was removed from the bath between E and F and slit open longitudinally from its mesenteric surface in order to exclude the possibility that the nicotine inhibitions result from passive lengthening of the longitudinal muscle by contractions of the circular layer.

Antagonism of the nicotine-inhibitions by ephedrine.—The inhibitory action of small doses of nicotine on the poisoned intestine resembles an adrenaline effect (Fig. 9), and the possibility that the ganglia activated by the nicotine are the cell

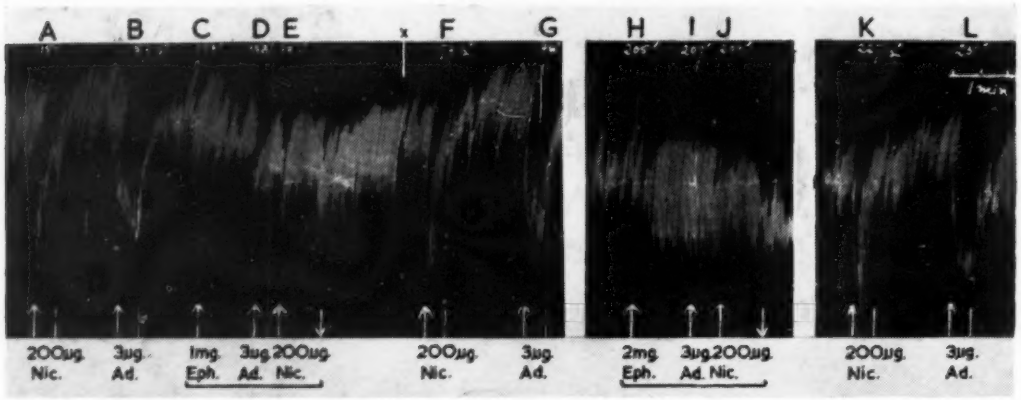

FIG. 9.—Continuation of Fig. 7. Effect of large doses of ephedrine (at C and H) on the responses to 200 µg. nicotine and to 3 µg. adrenaline respectively. At C to E, and at H to J, ephedrine, adrenaline, and nicotine were given in succession without washing out in between (for details see text). Drum stopped at X.

bodies of short adrenergic neurones suggests itself. This possibility could be tested by examining whether the inhibitory effect of these small doses of nicotine is, like the adrenaline effect, abolished by high concentrations of ephedrine. The antagonism of adrenaline actions by ephedrine was described by Schaumann in 1928 and also by Curtis in 1929. Finkleman (1930) observed that, in concentrations of $0.5–1 \times 10^{-4}$, ephedrine had a parallel action in abolishing the effect of splanchnic-nerve stimulation on the rabbit's intestine.

In five experiments the effect of ephedrine on the botulinized gut was examined in concentrations varying between 0.5 and 2×10^{-4}. In all five the inhibitory response to small doses of nicotine was abolished, provided the ephedrine was left in the bath for over 60 seconds; after shorter exposures to ephedrine the extinction of the inhibitory effect of nicotine was sometimes incomplete. In the experiment of Fig. 9, a concentration of 1×10^{-4} of ephedrine reduced considerably, but did not completely abolish, the inhibitory effects of adrenaline and of nicotine (at C to E), but later in the same experiment complete extinction of the two parallel effects was achieved (at H to J) by doubling the concentration of ephedrine. In another experiment (Fig. 10) the higher concentration (2×10^{-4}) of ephedrine itself caused inhibition, which, however, showed no further increase when nicotine was added to the bath in its presence (Fig. 10 at C). When the ephedrine was washed out and its own inhibitory effect had passed off, the intestine, which was again exhibiting strong

pendulum movements, remained for some time insensitive to the inhibitory action of nicotine (Fig. 10 at D).

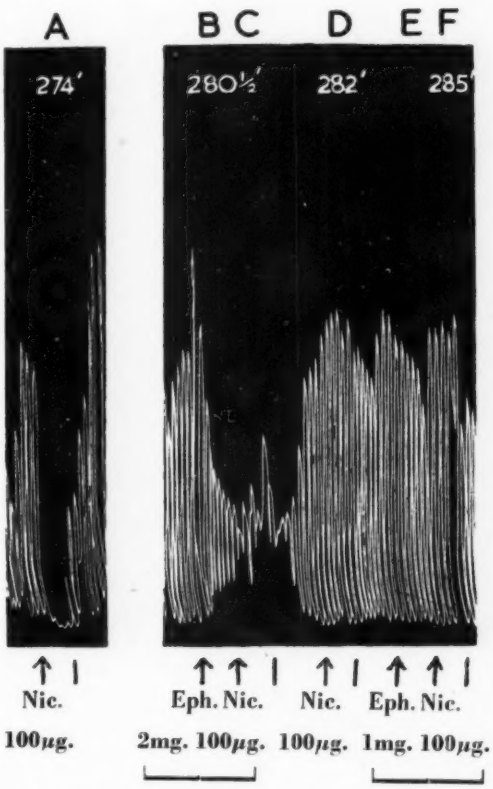

FIG. 10.—Ileum of rabbit (1.4 kg.) injected with 8 mg. of botulinum toxin A_1. Injection at both ends of intestinal segment. Death after 3¼ hours. The minutes on top of the tracing refer to time after death. Effect of 2 mg. (at B) and 1 mg. (at E) of ephedrine on the responses to 100 μg. nicotine (at A, C, D, and F). At C and F, nicotine given before washing out the ephedrine (for details see text.)

DISCUSSION

The results demonstrate the selective action of botulinum toxin upon certain elements within the nerve plexuses of the intestine. The disappearance of the motor response to nicotine in the poisoned gut serves to locate the paralysis in those post-ganglionic fibres which probably constitute a final common path not only for augmentor vagal impulses but also for the motor component of the myenteric reflex. These post-ganglionic neurones are generally considered as cholinergic, and their susceptibility to botulinum toxin is therefore entirely according to expectation (see Ambache, 1951).

The centre of interest shifted at once when inhibitory responses to nicotine began to appear in the poisoned preparations, because they seemed to reveal a new aspect of the complex neural organization of the enteric plexuses. It is believed that the present findings may fall into line with certain other inhibitory phenomena which have been observed previously by various workers, both in physiological and in pharmacological experiments on mammalian intestines, and that the explanation of them may have a common neurological basis.

In 1899 Bayliss and Starling showed that the peristaltic reflex comprises two distinct components: a motor component above the bolus, and an inhibitory component, consisting of active relaxation of the intestine, below the bolus. This preceding wave of inhibition is abolished by paralysing doses of nicotine and is therefore also of nervous origin. Thus there seems to be little doubt that the organization of the peristaltic reflex centres provides yet another example of reciprocal innervation. That being so, it is not unnatural to suppose that inhibitory neurones are present in one or both enteric plexuses. It is also reasonable to assume that these neurones are accessible both to nervous impulses impinging upon them from the afferent limb of the peristaltic reflex arc (or from the vagus) and to the influence of drugs which stimulate the ganglion cells of the autonomic nervous system.

These conclusions, arrived at on purely theoretical grounds, receive some support from histological findings. Certain observations published by Kuntz (1922) are

consistent with such a concept of a functional duality within the myenteric plexuses. Kuntz drew attention to the existence of two separate types of ganglion cell which were found together, within the same ganglion, either in the myenteric or the submucous plexus. The cells appeared to lie in close spatial proximity to each other, and were distinctive both in their staining properties and in their mutual connexions. These cell-pairs are shown in Fig. 11, which is a reproduction from Kuntz's paper. One member of the pair is stated to stain intensely with methylene blue, and sends out a short process ending in numerous synaptic terminations surrounding the other ganglion cell, which stains lightly or not at all. In attempting to correlate function with structure, it is worth speculating whether this anatomical arrangement typifies the duality of these nerve-centres and is at the basis of the reciprocal phenomena in the intestine.

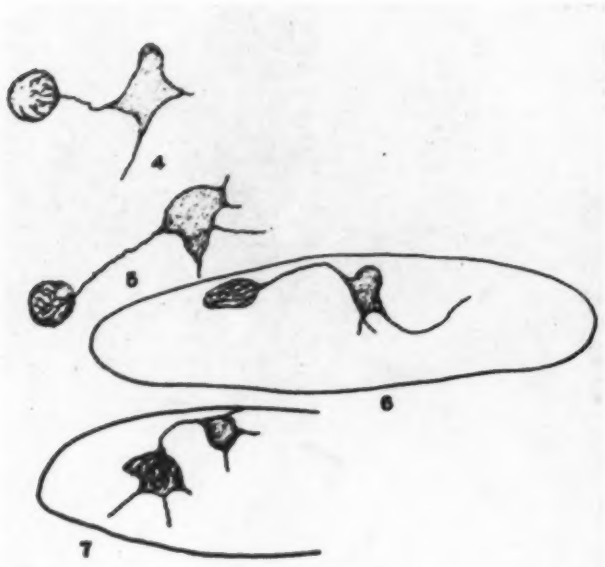

FIG. 11.—Paired arrangement of nerve-cells in enteric ganglia, reproduced from Kuntz (1922), *Anat. Rec.*, **24**, 199.

The present experiments provide physiological evidence for the existence in these nerve-centres of ganglion cells which, when stimulated by nicotine, are capable of producing inhibition in the intestine, and the histological fiindings of Kuntz may provide the structural basis for this function. Moreover, these ganglion cells appear to be the cell bodies of short adrenergic neurones. This conclusion is based on the finding that the inhibitory function is abolished by ephedrine, which suggests that the nerve fibres arising from these cells act on the smooth muscle by releasing adrenaline, or a mixture of adrenaline and *nor*adrenaline, i.e. sympathin. On this interpretation it is necessary to assume that both types of ganglion cell found by Kuntz send fibres to the smooth muscle, apart from the fact shown in Fig. 12 that one may be internuncial to the other.

Next we must consider whether these inhibitory neurones behave as isolated units subserving a purely local reflex function or whether they are linked up in any way with the extrinsic innervation of the gut. The latter seems more probable as there are good experimental grounds for supposing that this inhibitory pathway is also accessible to vagal impulses in certain species, for instance in cats and dogs. Even under normal conditions, in which the motor effect is predominant, signs of underlying inhibition on vagal stimulation can be detected according to Bayliss and Starling. In addition, after atropine, stimulation of the vagus can produce pure inhibition in the stomach and intestines of cats and dogs (Bayliss and Starling,

1899, 1901; McSwiney and Robson, 1929), because atropine abolishes the cholinergic motor effect, and so allows the stimulation of the inhibitory nerve-cells to be revealed.

The presence of both motor and inhibitory neurones in the gut was clearly envisaged by Langley in 1922 when he published his interesting diagram featuring two vagal pathways, one motor and the other inhibitory, each of which, in the intestine, is distinct throughout. His conception was that the vagus contains two sets of preganglionic nerve fibres, each impinging upon a distinct group of intermediate ganglion cells (his " vagus cells "), and that each of these in turn innervates a distinct set of terminal ganglion cells (his " local cells "), one set supplying motor and the other inhibitory fibres to the peripheral musculature. Langley thus postulates a succession of three neurones in both peripheral pathways of the vagus, the last two neurones being situated in the intestinal wall. The terminal neurones are of two types, either motor or inhibitory, and are in turn innervated by penultimate neurones of the appropriate type.

Since the present experiments do not throw any light on the problem of the connexion of the vagal fibres with the different ganglion cells, the " vagus cells " of Langley have been omitted from the diagram in Fig. 12 for the sake of clarity, the two types of " local cells " postulated by Langley being represented as the cell bodies of cholinergic and adrenergic neurones respectively. The diagram further

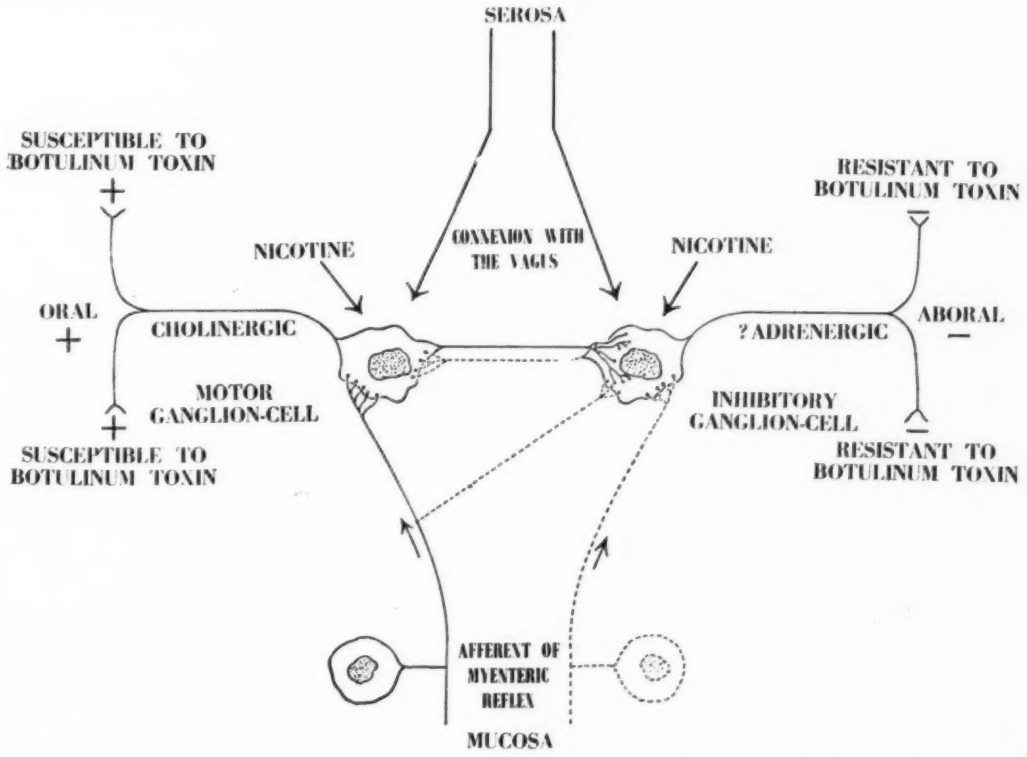

FIG. 12.—Diagram of the suggested connexions, within the enteric plexus, which would account for the reciprocal antagonism in the peristaltic reflex, for the dual effect of the vagus in certain species, and for the pharmacological actions of nicotine. (For explanation see text.)

indicates that nicotine is able to stimulate both types of " local cells." Usually, the predominant effect is that of the motor cells, whether they be excited by nicotine or by vagal impulses, although there are occasional signs that the inhibitory cells are stimulated as well. In order, however, to unmask the function of these inhibitory neurones, the motor set of neurones has to be inactivated; this is achieved with botulinum toxin, which eliminates the response of the final motor neurones by paralysing their cholinergic terminations. Previous experiments on the iris and on the nictitating membrane (Ambache, 1949, 1951) have proved that botulinum toxin has a selective affinity for nerve-endings of the cholinergic variety, but that it spares other types of nerve-endings in the same tissue, for instance those that are adrenergic. The appearance in the poisoned preparation of inhibitory responses to nicotine, which are the result of ganglionic stimulation of what are probably adrenergic neurones, is easily understood if we assume that botulinum toxin produces the same kind of differential denervation in the intestine as in the iris. In the diagram the susceptibility of the cholinergic nerve terminals to botulinum toxin and the resistance of the adrenergic terminals are indicated. It will be clear from this diagram why nicotine, acting on both types of ganglion cell, can exert only an inhibitory effect on the botulinized gut once the cholinergic neurones have succumbed. This unmasking action illustrates the kind of use to which botulinum toxin may be put in the analysis of nervous phenomena involving a mixture of cholinergic and non-cholinergic neurones.

The diagram also illustrates the participation of the two types of terminal neurones in the peristaltic reflex and includes another feature which may help the reader to visualize the unidirectional polarity of this reflex. It is only necessary to assume that the two sets of terminal neurones are orientated in the axis of the intestine in such a way that the cholinergic motor axons are directed orally, and the adrenergic inhibitory axons aborally, to account for the contraction above the bolus and for the phenomenon of " descending inhibition " described by Bayliss and Starling. Lastly, an attempt has been made to represent also the sensory side of the peristaltic reflex arc. It is not certain whether each type of ganglion cell commands a separate sensory innervation. In view of the existence of a collateral connexion between the two terminal neurones, a connexion to one type is all that is needed to complete the arc. But the possibility of a separate sensory connexion to the inhibitory ganglion cell is shown by dotted lines.

The presence, locally, of adrenergic neurones makes it necessary, in the analysis of the effects of certain pharmacologically active substances on the intestine, to take into consideration the possibility that stimulation of this type of ganglion cell contributes a component to the final response of the intestine. For it is possible to imagine that various known ganglion-cell stimulating agents (e.g., KCl, $BaCl_2$, etc.) can activate the two antagonistic types of local nerve cells and that a different balance may be struck between the two possible (motor and inhibitory) effects according to the substance or the dose thereof.

Summary

1. Loss of peristaltic activity and of the motor response to nicotine has been observed in isolated intestinal preparations taken from mice injected with botulinum toxin and showing symptoms of systemic botulism.

2. Botulinum toxin has been injected into the wall of the small intestine of rabbits in order to produce a high local concentration of the toxin in the intestinal wall ; a few hours later the poisoned intestinal preparation was examined in an organ bath. It showed its normal response to acetylcholine or eserine, but a gradual loss of the stimulating action to small doses of nicotine; these produced instead inhibition.

3. The inhibitory nicotine response of the intestinal preparation subjected to botulinum toxin is, like the stimulating response of the normal preparation, due to stimulation of ganglion cells, as it disappears after large, paralysing doses of nicotine and after hexamethonium; i.e., after paralysis of the ganglion cells of the myenteric plexus.

4. The inhibitory response to small doses of nicotine is abolished by large doses of ephedrine, which abolish the inhibitory action of adrenaline. It is therefore concluded that the inhibitory response to small doses of nicotine results from stimulation of ganglion cells which are the origin of short adrenergic neurones.

5. The change in the nicotine response of the rabbit's intestine after local intoxication with botulinum toxin is explained by the selective affinity of the toxin for cholinergic nerve-endings; this leads to abolition of the stimulating action of small doses of nicotine; at the same time it unmasks the response of ganglion cells which give rise to adrenergic fibres, since the latter are spared by the toxin.

6. Evidence is thus presented that there are two kinds of functionally distinct ganglion cells in the myenteric plexus. Stimulation of the one, which gives rise to cholinergic fibres, causes contraction; stimulation of the other, which gives rise to adrenergic fibres, causes inhibition of the intestine. The role of the two different types of ganglion cells in the peristaltic reflex is discussed.

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ANALYSIS OF CERTAIN INTERACTIONS OF NICOTINE WITH BRADYKININ AND HISTAMINE

BY

N. AMBACHE* AND M. ROCHA e SILVA

From the Department of Biochemistry and Pharmacodynamics, Instituto Biologico, São Paulo, Brazil

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The method of using nicotine to produce paralysis of autonomic ganglia was first introduced by Langley in 1890. Since then this drug has been used from time to time as an analytical weapon in pharmacological studies on smooth muscle, in attempts to distinguish between substances which act directly on the muscle fibres and drugs which produce contraction by the stimulation of local ganglion cells. Unfortunately this analysis is often complicated by the following factors:

(*a*) The possibility of a direct antagonism between nicotine and some drugs but not others. This antagonism is observed when large doses of nicotine are kept in the bath in the presence of the stimulating agent. For instance, as we shall show, large doses of nicotine suppress the effect of histamine but not that of bradykinin. We believe that this antihistamine effect is a phenomenon of genuine antagonism; it is short-lived, and it usually lasts only as long as the nicotine is in the bath.

(*b*) The fact that nicotine is also an inhibitor of cholinesterase (Nachmansohn, 1939).

(*c*) The existence of an after-effect following large contractions evoked by smaller stimulant doses of nicotine, a phenomenon which we propose for the moment to call " residual inhibition." The nature of this inhibition is at present not quite clear. It bears some resemblance to a type of after-inhibition produced by other drugs, which was described by Cantoni and Eastman (1946) and was later re-studied by Beraldo and Rocha e Silva (1949). These authors observed a refractory state occurring after maximal contractions induced by acetylcholine and by pilocarpine, and lasting, in the guinea-pig's ileum, for as long as 10–15 min. afterwards. It was suggested by the authors quoted that the contraction produced an exhaustion of some essential energetic metabolite. It is likely, as suggested by Emmelin and Feldberg (1947), that the phenomenon which we have called " residual inhibition," and which was also observed by them after a stimulant dose of nicotine, may owe its origin to a similar process.

The antagonism of nicotine to histamine when the two are present together was observed earlier by Bernheim (1933), who came to the conclusion that the two drugs were " acting on the same receptor substance in the intestine." More recently Feldberg (1950) has stated that, apart from producing ganglionic block,

* On leave of absence from the Ophthalmological Research Unit, Medical Research Council, Institute of Ophthalmology, Judd Street, London, W.C.1.

large doses of nicotine " reduce the excitability of the muscle fibres as seen from the strongly reduced responses to histamine." In considering these two statements it seemed necessary to clarify the points (i) whether or not the antihistamine effect of nicotine was associated with a general reduction in excitability to all drugs, and (ii) whether it indicated a depression of the contractile mechanism. To put this matter to the test, we have availed ourselves of bradykinin, a newly described active principle, recently isolated by Rocha e Silva, Beraldo, and Rosenfeld (1949). This is a " slow " smooth-muscle-contracting substance, derived from plasma globulins by the proteolytic action of the venom of snakes of the genus *Bothrops* or of trypsin. We have found that, once the ganglion cells are paralysed by large doses of nicotine, the presence of the latter in the organ bath does not diminish the size of contractions produced by bradykinin; this result testifies to the absence of muscular depression by nicotine in concentrations of up to 3×10^{-4}. In contrast, as mentioned already, histamine contractions are suppressed by the same large amounts of nicotine. This distinction between histamine and bradykinin and the nature of the antihistamine and after-inhibitory actions of nicotine have been our main interests in the present experiments.

Methods

The preparation of guinea-pig's ileum was made in the usual way; it was attached to a stainless steel-wire strut and suspended, with its lumen open at both ends, in a 7-c.c. organ bath filled with Tyrode's solution, and oxygenated with air. Contractions were produced at regular intervals (indicated in the Figures) by means of two " testing drugs," histamine or bradykinin, and were recorded with a compensated linear frontal-writing lever (Schild, 1947). Nicotine was not introduced into the bath until a " steady level " was attained in the response to the testing drugs. Drugs were added in a volume of 0.05–0.3 c.c. (rarely 0.5 c.c.) of distilled water. The preparation of bradykinin has been described before (Rocha e Silva, Beraldo, and Rosenfeld, 1949); different batches of " crude " bradykinin with an activity of about 1 " unit " per mg. (Prado, Beraldo, and Rocha e Silva, 1950 were used. The dilutions of nicotine were made from the free base.

Results

Ganglion-cell paralysing concentrations of nicotine (3×10^{-4})

Our procedure was devised to maintain the ganglion cells in a state of paralysis throughout the experiment by the administration of nicotine at intervals. A standard dose of nicotine (2 mg.; referred to as the " paralysing dose ") was kept in the bath for at least one minute, only the first dose producing a short phase of stimulation followed by paralysis, and occasionally by a contraction just after the removal of nicotine by washing (asterisks in Figs. 4 and 5). Time was allowed for the subsidence of this " wash-out contraction " before retesting the gut with a second or third dose of 2 mg. nicotine, neither of which elicited contraction. On the contrary, these repeat doses appeared to hasten relaxation after the " wash-out contraction." Subsequently, the 2-mg. dose of nicotine was repeated from time to time, without producing contractions either on introduction or after the wash-out, as shown in Figs. 2 and 3.

Bradykinin.—When the intestine was treated with these paralysing doses of nicotine, and bradykinin was added subsequently, either before or after removal of

nicotine from the bath, there was no significant decrease in the response to bradykinin. This is shown quite clearly in Figs. 1 and 2. Only once in ten trials (three experiments) was the response to bradykinin depressed in the presence of the paralysing dose of nicotine. In thirteen other trials (also in three different experiments), in

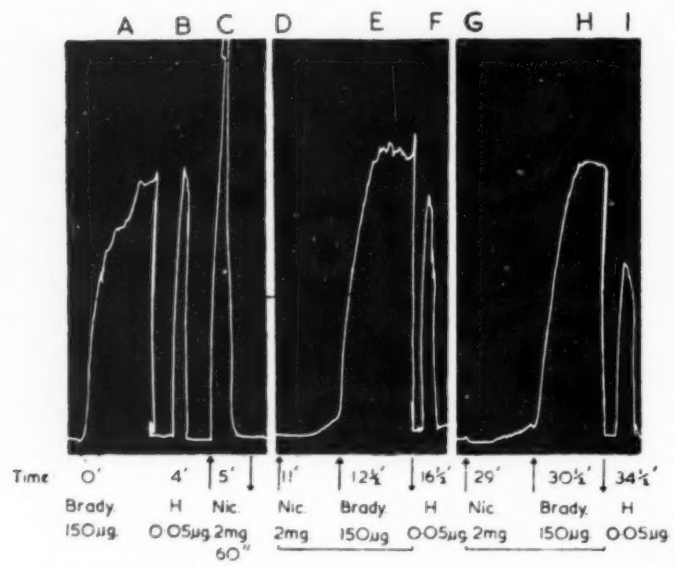
A B C D E F G H I
Time 0' 4' 5' 11' 12½' 16½' 29' 30½' 34½'
Brady. H Nic. Nic. Brady. H Nic Brady. H
150µg. 0·05µg. 2mg. 2mg. 150µg. 0·05µg. 2mg. 150µg. 0·05µg.
60"

FIG. 1.—Guinea-pig ileum in 7-c.c. bath. ↑ indicates addition of drug, ↓ indicates that the solution in the bath was changed. The first dose of nicotine, at C, produced a powerful contraction followed rapidly by relaxation. Subsequent doses at 6½ and 18 min. (not shown; both left in the bath for 2 min.) and at D and G had no such effect, indicating the persistence of ganglion-cell paralysis throughout the experiment. The effect of 150 µg. of bradykinin (at E and H) in the presence of nicotine was no smaller than its initial effect at A in the absence of nicotine. The experiment shows that a large dose of nicotine *per se* does not depress the contractile mechanism of intestinal muscle.

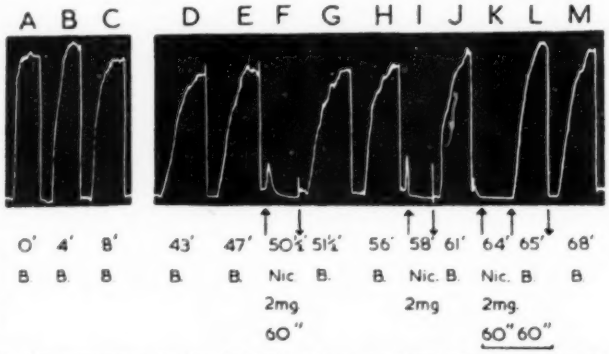

FIG. 2.—Preparation as in Fig. 1. Muscle tested with a standard dose of 50 µg. bradykinin for 60 sec. throughout. A, B, C, initial response to bradykinin. Between C and D the ganglion cells were paralysed with 2 mg. nicotine (at 11 min.; not shown). This paralysis was maintained by frequent repetition of the same dose of nicotine at intervals for the remaining 1¼ hours of the experiment. During this time there did not appear to be any significant difference between (*a*) the response to bradykinin in the presence of nicotine (at L); (*b*) the response to bradykinin after, or just after, a dose of nicotine had been washed out (at G, J, and M); and (*c*) the response to bradykinin alone.

which the bradykinin was added *after* washing out the paralysing dose of nicotine, only twice was there any detectable after-inhibitory effect upon bradykinin, while eleven times no trace of inhibition could be detected. These experiments point to the conclusion that the contractile mechanism in the plain muscle of the guinea-pig intestine is not affected by these doses of nicotine.

Histamine.—In contrast with the resistance of the bradykinin-response to nicotine, the action of histamine was definitely affected by the paralysing doses of nicotine when the two were present together in the organ bath. Without exception, 2 mg. nicotine produced a strong reduction in the response to a test dose of histamine, or even its suppression (Fig. 3). This antagonism was observed in nine out of nine

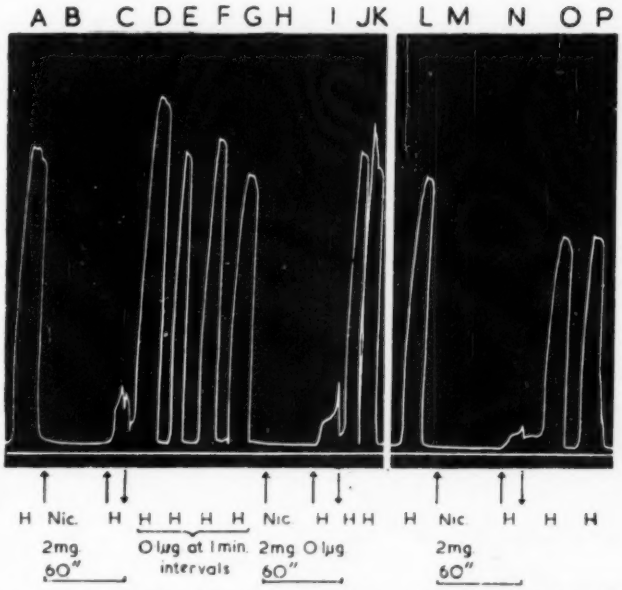

FIG. 3.—Preparation as in Fig. 1. The record illustrates the direct antagonism of a large dose (2 mg.) of nicotine to histamine. The ganglion cells were paralysed with 2 mg. nicotine some time before A, and were kept paralysed throughout the experiment by repeating the nicotine at frequent intervals. The effect of a standard dose of histamine (0.1 μg.) was antagonized by the presence of nicotine in the bath, at C, I, and N. After removal of the nicotine the histamine effect recovered at D, and again at J and O, within 1 minute of the nicotine being washed out. Bradykinin was not antagonized by the same dose of nicotine earlier in this experiment.

trials (two experiments). It may be tentatively considered as a "competitive" type of phenomenon, because the antihistamine effect disappeared quite soon after the nicotine had been washed out (Fig. 3, D, J, and O), although the ganglion cells remained in a paralysed state for a considerable time thereafter, as shown by the ineffectiveness of further doses of nicotine repeated at intervals. Thus, in contrast with what happened after stimulating doses of nicotine (see next section), there was little or no residual inhibition after paralysing doses. After the removal of nicotine, the muscle may regain its full sensitivity to histamine within 60 sec. (Fig. 3, J), but a slight depression of the histamine response after the withdrawal of nicotine was observed on one or two occasions (for instance, Fig. 1 at I).

Stimulating doses of nicotine (100–150 μg.)

A small dose of nicotine (10 μg.), although capable of evoking a contraction, was not followed by a decrease of subsequent responses to histamine. A larger stimulant dose (100 to 150 μg.) produced both effects—first the contraction, and subsequently, after the removal of nicotine, a variable reduction in the response to successive test-doses of histamine. This phase of post-nicotine "residual inhibition" is of several minutes' duration. The time-course of recovery and the shape of the inhibitory curve after nicotine varied slightly. For instance, in Fig. 4 the second histamine-contraction (at F) after the first dose of nicotine was considerably more depressed than the preceding one at E (cf. Beraldo and Rocha e Silva, 1949; Fig. 4A). But, after the next 150-μg. dose of nicotine at N, the first histamine-contraction, at O, was more depressed than the second at P. Allowing for this variability, the curve described by this inhibitory effect resembled, in many instances, that of the Cantoni and Eastman (1946) phenomenon alluded to in the introduction. This type of "residual inhibition" after 100–150-μg. doses of nicotine was observed regularly in a total of sixteen out of sixteen trials, in six experiments on different preparations.

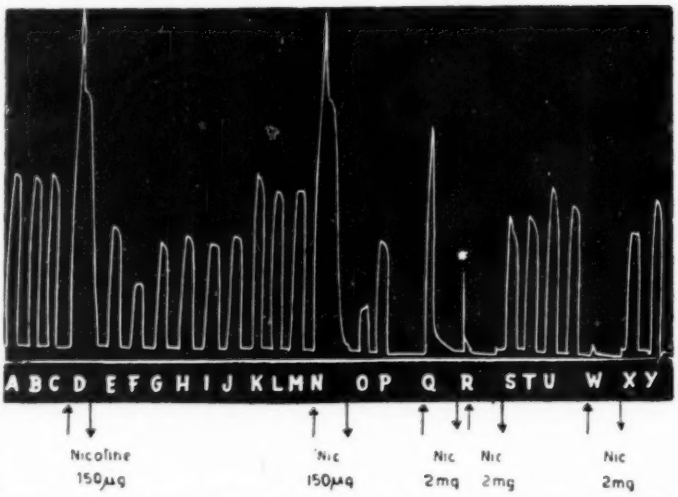

FIG. 4.—Preparation as in Fig. 1. With the exception of the responses to nicotine, which was left in the bath for 60 sec., all contractions were produced by a standard dose of histamine (0.1 μg.) every 1–2 min. The experiment illustrates: (1) the persistence of residual inhibition for 11 min. (E–K) after the first stimulant dose (150 μg.) of nicotine; (2) further inhibition after another 150 μg. of nicotine at N. Two paralytic doses (2 mg.) of nicotine, at Q and R, were followed by smaller inhibitions (S, T, and U), as was a third dose (at W), although the dose of nicotine was more than 10 times greater than at D or N. The contraction marked by an asterisk occurred after the wash-out following Q, and before R; time was allowed for its subsidence.

The " residual inhibition " produced by successive stimulant doses of nicotine is additive; thus, in Fig. 5 the second 100-μg. dose of nicotine at F was so spaced as to fall in the recovery period after the first at B (and J in that of F); each dose was followed by an increase in the depth of inhibition produced by its predecessor.

" Residual inhibition " can be distinguished from the so-called competitive type of antagonism between large doses of nicotine and histamine described above. The two processes appear to be quite different for the following reasons: (i) whereas the " antagonism " lasts only as long as nicotine is in the bath, the " residual "

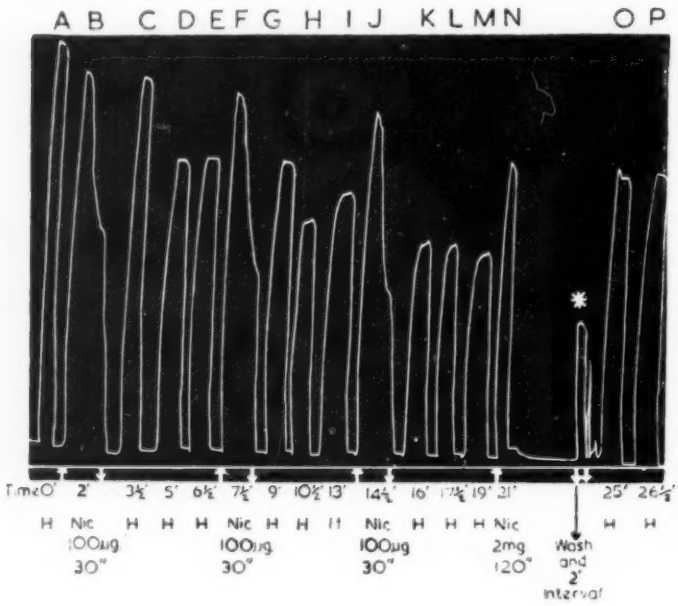
A B C D E F G H I J K L M N O P
Time 0' 2' 3½' 5' 6½' 7½' 9' 10½' 13' 14½' 16' 17½' 19' 21' 25' 26½'
H Nic H H H Nic H H H Nic H H H Nic H H
100μg 30" 100μg 30" 100μg 30" 2mg 120"
Wash and 2' interval

FIG. 5.—Preparation as in Fig. 1. Muscle tested with a standard dose (0.15 μg.) of histamine at regular intervals every 1½ min. Summation of residual inhibition produced by successive stimulant doses of 100 μg. nicotine for 30 sec. at B, F, and J. A paralytic dose of 2 mg. nicotine for 120 sec. at N appears to produce less residual inhibition (at O and P). The contraction marked with an asterisk occurred after the nicotine had been washed out. An interval of 2 min. was allowed for its subsidence.

effect is of long duration, lasting several minutes after the removal of nicotine, and may take some time before it reaches its maximum ; and (ii) the residual inhibition could not be observed after previous paralysis of the ganglion cells; and, as shown in Figs. 4 and 5, when the effect of small and large doses of nicotine was examined successively, the large dose appeared to produce less residual inhibition than the small dose (e.g., Fig. 4, X).

Discussion

On the basis of the experiments presented in this paper the following conclusions can be reached. Firstly, nicotine in concentrations of up to 3×10^{-4} (*a*) has no depressing effect upon the contractile mechanism in the smooth muscle of the guinea-pig's ileum, and (*b*) does not affect its excitability and responsiveness to all drugs. This point is clearly demonstrated by the fact that large doses of nicotine did not reduce the size of the contractions evoked by bradykinin, even when the two drugs were present together.

Secondly, interaction between nicotine and histamine is of two kinds. There is, first, the antagonism of the large dose of nicotine to histamine, when the two are present together. Since we have excluded (with bradykinin) the possibility of a harmful effect of nicotine upon the contraction process proper, this second effect of nicotine upon histamine-contractions must receive some other explanation. Since this antagonism disappears rapidly almost as soon as the nicotine is washed out, it appears to originate in a phenomenon of a " competitive " nature.

The second type of interaction between nicotine and histamine is the so-called " residual inhibition " of histamine, which is seen for several minutes after the smaller stimulating dose (100–150 μg.) of nicotine. This may be an after-effect of the contraction itself (or of the release of acetylcholine in the gut which led to the contraction), rather than a primary effect of the nicotine, since the larger " paralysing " dose of nicotine produced less residual inhibition. The mechanism of this " residual inhibition " raises a further point of theoretical interest, namely whether the ganglion cells play any part in this and allied phenomena. If the explanation proposed by Cantoni and Eastman (1946) for the phenomenon which they observed be accepted, then a temporary exhaustion of an essential energetic metabolite after the contraction produced by the stimulating dose of nicotine might also be assumed; this " residual inhibition " would then be comparable to that produced by any maximal contraction set off by such distinct substances as acetylcholine, histamine, and pilocarpine. Paralysis of ganglion cells appears to play no role in the production of this long-lasting residual inhibition after nicotine, since the larger paralysing dose of nicotine appeared to produce less residual inhibition than the smaller stimulating doses. That the Cantoni-Eastman effect is not itself due to ganglionic paralysis was further shown by the fact that it could still be obtained as a sequel to maximal contractions induced by strong doses of acetylcholine (10 and 20 μg. in two different experiments), in preparations of which the ganglion cells were previously paralysed with nicotine. One of these experiments is illustrated in Fig. 6. Although the ganglion-cell paralysis was maintained between A and F (as tested again with 2 mg. of nicotine at F, which maintained paralysis thereafter), the refractoriness towards histamine, which is characteristic of the Cantoni-Eastman effect, developed as usual after the 20-μg. dose of acetylcholine.

FIG. 6.— Continuation of Fig. 5 after an interval of 5½ min. Refractory state after a large contraction produced by acetylcholine (Cantoni and Eastman effect). Muscle tested at intervals with a standard dose of histamine (0.15 μg.). Ganglion-cell paralysis was maintained by a 2-mg. dose of nicotine (not shown) administered 3 min. before A, and left in the bath for 2 min.; when repeated at F, this dose (2 mg. for 60 sec.) was ineffective, and the ganglion cells may be presumed to have been paralysed throughout the experiment, yet the refractory state which follows a large dose of acetylcholine (20 μg. for 60 sec. at B) developed as usual, and appears to be independent of ganglion-cell function.

SUMMARY

1. The smooth muscle in the guinea-pig's ileum is not depressed by concentrations of nicotine which are used to produce ganglion-cell paralysis (3×10^{-4}).

2. This was shown in experiments in which the intestine, maintained in a prolonged state of ganglion-cell paralysis by frequent repetition of 2-mg. doses of nicotine, showed no decrease in its response to bradykinin, either in the presence of nicotine or after its removal.

3. In contrast, once the ganglion cells are paralysed, the action of histamine is antagonized by the presence of a 2-mg. dose of nicotine in the bath, but shows recovery almost as soon as the nicotine has been washed out.

4. Stimulating doses of nicotine (100–150 μg. for 30–60 sec.), producing large contractions, leave in their wake a state of " residual inhibition " which may last up to 12 min. (Fig. 4). This appears to be an after-effect of the contraction, and resembles an effect described by Cantoni and Eastman (1946) after other contracting drugs.

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RESPONSES OF CORONARY VESSELS TO PITUITARY (POSTERIOR LOBE) EXTRACT AND TO ADRENALINE

BY

PAMELA KORDIK

From the Department of Pharmacology, University of Oxford

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During exercises in practical pharmacology for students, it has often been observed that pituitary (posterior lobe) extract and adrenaline produce unexpected changes in coronary flow. The observations have been made when the heart of the rabbit or of the cat has been perfused by Langendorff's method with oxygenated Locke's solution at 35°–37° C. Posterior lobe extract and adrenaline were injected into a cannula tied in the aorta through a rubber tube attached to a side arm of the cannula. The amounts of posterior lobe extract which were injected varied from 0.01 to 0.04 unit for rabbit hearts and from 0.02 to 0.1 unit for cat hearts. The amounts of adrenaline varied from 0.1 to 5.0 μg. for rabbit hearts and from 0.02 to 0.5 μg. for cat hearts. The rate of flow of Locke's solution coming from the heart was recorded continuously by Stephenson's recorder (1948).

The student is taught that posterior lobe extract constricts the coronary vessels. In the rabbit heart this constriction is almost invariably observed, but in the cat

TABLE I

EFFECT OF PITUITARY (POSTERIOR LOBE) EXTRACT ON CORONARY FLOW

Rabbit heart					Cat heart				
Exp.	No. of observations	Constriction	Constriction followed by dilatation	Dilatation	Exp.	No. of observations	Constriction	Constriction followed by dilatation	Dilatation
1	2	1	1	—	10	6	—	—	6
2	5	5	—	—	11	3	—	—	3
3	2	2	—	—	12	3	3	—	—
4	3	3	—	—	13	6	6	—	—
5	3	3	—	—	14	8	—	4	4
6	4	4	—	—	15	8	1	—	7
7	2	2	—	—	16	7	—	7	—
8	3	3	—	—	17	7	3	4	—
9	3	3	—	—	18	5	—	3	2
Total	27	26	1	0	Total	53	13	18	22

heart the constriction is often followed by dilatation, or dilatation may be the only response. In Table I the results of nine experiments on rabbit hearts and of nine experiments on cat hearts are recorded. It will be seen that the rabbit heart responded to posterior lobe extract according to expectation, but that the cat heart did not. In the cat heart dilatation was the only effect seen in Exps. 10 and 11, whereas constriction was the only effect in Exps. 12 and 13. In Exp. 14 the total number of injections was eight, of which four produced initial constriction followed by dilatation, while four produced dilatation alone. Similarly in Exp. 17 the total number of injections was seven, all of which produced constriction, but four of the injections produced dilatation after the constriction. The totals at the foot of Table I show that in the cat dilatation is more commonly produced by posterior lobe extract than is constriction (see Fig. 1).

FIG. 1.—Isolated cat heart. Upper record is the coronary flow; dilatation was recorded upwards. Lower record is amplitude of beat. Figures below the record are heart rate per min. At the arrow 0.02 unit pituitary (posterior lobe) extract was injected into the aortic cannula. Note the coronary dilatation.

FIG. 2.—Isolated rabbit heart. Records as in Fig. 1. At the arrow 0.5 μg. adrenaline was injected. Note the coronary constriction.

When adrenaline is injected there is a change in the force of the contraction which may modify the coronary flow by obstructing it; in consequence it is less easy to discover the direct effect of adrenaline on the coronary vessels. Table II shows the results in experiments on nine rabbit hearts and ten cat hearts. In the rabbit, adrenaline caused constriction in almost all of the fifty-seven trials, while a succeeding dilatation was observed in one-third of them (see Fig. 2). In the cat initial constriction was caused by adrenaline in less than one-fifth of the trials, while dilatation was observed sixty-two times out of sixty-three.

TABLE II

EFFECT OF ADRENALINE ON CORONARY FLOW

Rabbit heart					Cat heart				
Exp.	No. of observations	Constriction only	Constriction followed by dilatation	Dilatation only	Exp.	No. of observations	Constriction only	Constriction followed by dilatation	Dilatation only
1	3	—	3	—	10	15	—	—	15
2	8	4	4	—	11	7	1	—	6
3	8	7	1	—	12	6	—	3	3
4	7	3	4	—	13	5	—	3	2
5	6	4	2	—	14	5	—	1	4
6	9	7	2	—	15	5	—	1	4
7	6	4	2	—	16	6	—	1	5
8	4	4	—	—	17	7	—	2	5
9	6	5	1	—	18	5	—	—	5
					19	2	—	—	2
Total	57	38	19	0	Total	63	1	11	51

The action of ephedrine.—The opportunity was also taken in these experiments to examine the effect of ephedrine in cat and rabbit hearts. Ephedrine increases the rate and amplitude of beat of the cat heart when injected in amounts from 5 to 20 μg., but it has little or no effect on the rate and amplitude of the rabbit heart even when injected in amounts ten times greater; thus in six of ten experiments ephedrine had no effect on the rate and amplitude of the rabbit heart, and in three of ten experiments it had little effect; only in one heart was the effect of ephedrine definite. On the coronary flow the effect of ephedrine was qualitatively similar to that of adrenaline in both cat and rabbit hearts, though its effect was more prolonged.

Discussion

When the coronary flow in the dog is investigated by means of a Morawitz cannula in the heart-lung preparation, adrenaline always increases the flow and posterior lobe pituitary extract always diminishes it. This contrast is much less sharp in the isolated heart of the rabbit and of the cat perfused with Locke's solution. Adrenaline causes constriction in the rabbit heart whereas posterior lobe extract causes dilatation in the cat heart. Evidently the contrast is at least as great between the coronary vessels of the rabbit and those of the cat as it is between the two agents. The coronary vessels of the rabbit respond much more by constriction to both agents, while those of the cat respond much more by dilatation. The same difference was shown by Gunn (1926) to hold good for histamine.

Summary

1. Pituitary (posterior lobe) extract very often causes dilatation of the coronary vessels of the isolated cat heart perfused by Langendorff's method. This dilatation may occur without antecedent constriction.

2. Adrenaline causes constriction of the coronary vessels of the perfused rabbit heart, dilatation following the constriction in only one-third of the trials.

3. Ephedrine stimulates the rate and amplitude of the cat heart, but is almost always without effect, in ten times the amount, on the rabbit heart.

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THE NATURE OF UTERINE AND INTESTINAL SYMPATHIN

BY

MONICA MANN AND G. B. WEST

From the Pharmacological Laboratories, School of Pharmacy, University of London, and Medical School, University of St. Andrews, Dundee

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Evidence is accumulating to show that a substance with the physiological properties of synthetic *l-nor*adrenaline is liberated together with adrenaline into the blood stream when certain adrenergic nerves in the cat are stimulated. Bülbring and Burn (1949) found that splanchnic nerve stimulation in spinal cats resulted in the liberation of varying proportions of *nor*adrenaline and adrenaline; their results were obtained in single animals by comparing the height of contraction of the denervated nictitating membrane with that of the normal membrane. Gaddum and Lembeck (1949) tested the blood from the suprarenal vein (after stimulation of the splanchnic nerve) on the rat's uterus and colon and confirmed this result. West (1950) injected the blood immediately after withdrawal from the suprarenal vein of one cat into the arteries supplying the nictitating membrane and non-pregnant uterus of a second cat under chloralose and cocaine, and obtained a similar result.

In a study of the nature of splenic sympathin, Peart (1949) tested the blood from the splenic vein after nerve stimulation on the rat's uterus and colon, the perfused rabbit's ear and cat's spleen, and nictitating membrane or lower lid of the cat. All the evidence supported the view that the active material was *nor*adrenaline in a concentration of 50 to 500 mμg./ml., although smaller amounts of adrenaline were sometimes liberated. Mann and West (1950) estimated the concentrations of the two amines in the blood from the splenic and hepatic veins after stimulation of the respective nerves and showed that the main active constituent was *nor*adrenaline in both types of blood; this was present in the blood of the hepatic veins in a mean concentration of 0.48 μg./ml., and in the blood of the splenic veins in a mean concentration of 0.13 μg./ml. Smaller amounts of adrenaline were sometimes also liberated. Using a similar method, we have estimated the concentrations of the two amines in the plasma after stimulation of the hypogastric and inferior mesenteric nerves, utilizing the formula developed by Bülbring (1949).

METHODS

Collection of uterine blood.—Cats anaesthetized with chloralose were used. The left hypogastric nerve was dissected free from peritoneum and divided centrally just below the inferior mesenteric ganglion. Blood from the left ovarian vein was led through a cannula and reservoir to the femoral vein. When blood samples were required, the reservoir was replaced by a graduated centrifuge tube standing in ice. Heparin or chlorazol fast pink was used as the anticoagulant, and plasma samples were obtained by rapid centrifugation. In about half the experiments the adrenals were excluded from the circulation by ligatures.

Stimulation of the nerve was through platinum electrodes with an ordinary coil (Faradic stimulation at 7.5 cm. on 4 V.) and usually lasted a total time of one minute. It was found that a good yield of sympathin was obtained with four alternate periods of 15 sec. stimulation and 30 sec. rest. During this time and for the next two minutes the volume of blood collected was usually 3–4 ml. In some experiments a bulldog clip was attached to the left uterine vein after stimulation had occurred in an effort to increase the yield of sympathin in the ovarian vein. The control plasma was obtained by bulking samples secured before, and 15 min. after, stimulation.

Collection of intestinal blood.—Cats anaesthetized with chloralose were used. The inferior mesenteric nerve was dissected free from the artery and divided centrally. Blood in the inferior mesenteric vein was led through a cannula and small reservoir to the femoral vein. The remainder of the method was similar to that described above. The adrenals were excluded from the circulation in all these collections. A better yield of sympathin was sometimes obtained with four alternate periods of 5 sec. stimulation and 55 sec. rest; with this intermittent stimulation, about 3 ml. of blood was collected in 5 min.

Assay methods.—These were essentially the same as those used in a previous study (Mann and West, 1950). The pharmacological tests were the isolated uterus from a non-pregnant rat in dioestrus, the isolated rectum of a week-old chick, and the chronically denervated nictitating membrane of a cat under chloralose. As originally stated, as little as 4 per cent of one of the amines could be accurately measured in a mixture of the two.

Results

Parallel quantitative assays by different methods were carried out on the plasma obtained in each experiment. The estimates were taken in pairs and values for adrenaline and *nor*adrenaline calculated by Bülbring's formula. In a series of 13 experiments (Table I), estimates of the uterine sympathin gave a mean value of 0.037 μg. *l-nor*adrenaline per ml. plasma. Small amounts of adrenaline were present in all

TABLE I

CONCENTRATION OF UTERINE SYMPATHIN IN POST-STIMULUS SAMPLES OF PLASMA

Exp.	μg./ml. plasma		Exp.	μg./ml. plasma	
	*l-nor*adrenaline	*l*-adrenaline		*l-nor*adrenaline	*l*-adrenaline
1	0.035	0.004	8*	0.008	0.003
2	0.001	0.003	9*	0.100	0.010
3	0.064	0.002	10*	0.095	0.005
4	0.050	0.001	11*	0.078	0.005
5*	0.005	0.005	12	0.024	0.002
6*	0	0.005	13	0.020	0.009
7*	0	0.010			

* Adrenal vessels open in these experiments; tied in all other experiments.

samples, the mean value being 0.005 μg. per ml. plasma, but *nor*adrenaline was absent from two specimens (Nos. 6 and 7). In Experiments 3, 10, and 11, the "post-stimulus" samples and *nor*adrenaline stimulated the isolated uterus from a non-pregnant rat in early oestrus, thereby supplying additional evidence that the main active material in these cases was *nor*adrenaline. It is clear that a mixture of the two amines is liberated when the hypogastric nerve in the non-pregnant cat is

TABLE II

CONCENTRATION OF INTESTINAL SYMPATHIN IN POST-STIMULUS SAMPLES OF PLASMA

Exp.	μg./ml. plasma		Exp.	μg./ml. plasma	
	*l-nor*adrenaline	*l*-adrenaline		*l-nor*adrenaline	*l*-adrenaline
1	0.138	0.001	10	0.012	0.050
2	0.045	0.001	11	0.031	0.019
3	0.013	0.025	12	0.009	0.005
4	0.020	0.009	13	0.096	0.002
5	0.020	0.020	14	0.144	0.006
6	0.008	0.002	15	0.090	0.010
7	0.008	0.002	16	0.036	0.004
8	0.015	0.010	17	0.044	0.006
9	0.050	0.050	18	0.017	0.008

stimulated, and usually *nor*adrenaline predominates. It is hoped to identify the sympathin liberated in pregnant cats by this technique.

In a series of 18 experiments (Table II), estimates of the intestinal sympathin gave mean values of 0.044 μg. *l-nor*adrenaline and 0.013 μg. *l*-adrenaline per ml. plasma. In this series, as in the uterine one, adrenaline was found in all samples.

Discussion

*Nor*adrenaline was detected by Euler (1948) in the sympathomimetic extracts of various mammalian organs including the uterus and intestine. Results reported here confirm his observations. Bacq and Fischer (1947) were the first workers to suggest that the mediator in the cat uterus might be *nor*adrenaline and not adrenaline, although they compared the results of intravenous doses of the amines with those of nerve stimulation. By reinjection of the uterine blood, West (1950) failed to reach any conclusion as to the possible nature of uterine sympathin. Probably his test objects were not sensitive enough since the amounts liberated are much smaller than those found in the hepatic and splenic veins. The inhibitory activity of *nor*adrenaline on the non-pregnant uterus of the cat is about one-eighth that of adrenaline, and on intestinal loops both cause dilatation (Burn and Hutcheon, 1949), adrenaline being slightly the more active of the two. Our results indicate, therefore, that physiologically equiactive amounts of the amines are liberated in the uterus and intestine. These results also add weight to the findings of Blaschko (1942) that *nor*adrenaline is the probable precursor of adrenaline in the body.

Summary

1. Stimulation of the hypogastric and inferior mesenteric nerves in cats resulted in the appearance of *nor*adrenaline and adrenaline in the plasma.

2. Parallel quantitative assays showed that *nor*adrenaline was present in the blood of the ovarian vein in a mean concentration of 0.037 μg./ml. (13 experiments), with 0.005 μg. adrenaline per ml. plasma: in the blood of the inferior mesenteric vein, *nor*adrenaline was present in a mean concentration of 0.044 μg./ml. (18 experiments) with 0.013 μg. adrenaline per ml. plasma.

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THE ACTIVITY OF THIOSEMICARBAZONES ALONE AND IN COMBINATION WITH OTHER DRUGS IN EXPERIMENTAL CORNEAL TUBERCULOSIS

BY

R. J. W. REES* AND J. M. ROBSON

*From the Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1

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The discovery of the antituberculosis activity of a number of benzaldehyde thiosemicarbazones was first announced by Domagk and his co-workers in 1946. Their results have since been confirmed, both experimentally and clinically, by a number of investigators. Unfortunately the more recent clinical studies (Mertens and Bunge, 1950; Simmons, Hobson, Resnick, DeNicola, and Bennett, 1950) have shown that the thiosemicarbazones so far tested clinically may produce serious toxic effects which limit their usefulness. Nevertheless these compounds remain of considerable importance especially as it is possible that thiosemicarbazones may eventually be found whose action will not be associated with serious toxic effects. Another possibility is that satisfactory chemotherapy of tuberculosis may result from the combination of thiosemicarbazones with other substances. There are indeed several findings which suggest that the combination of chemotherapeutic substances may prove of value; for example, a preliminary report by the Medical Research Council (1950) demonstrated quite clearly the effectiveness of using *p*-aminosalicylic acid with streptomycin in order to reduce the emergence of streptomycin-resistant tubercle bacilli in man.

Other workers have tried a combination of a thiosemicarbazone with another substance in experimental tuberculosis. Hurni (1949) used a thiosemicarbazone (TB1/698) with streptomycin in mice, but did not find any increased beneficial effect; on the other hand Karlson, Gainer, and Feldman (1950) were able to demonstrate the value of such a combination in experimental tuberculosis in the guinea-pig. Ulstrup (1950) used a combination of the thiosemicarbazone TB1/698 with *p*-aminosalicylic acid in experimental tuberculosis in the guinea-pig, but found only a slight potentiation.

We have used the mouse corneal technique (Rees and Robson, 1950) to test the activity of a number of thiosemicarbazones, either alone or in combination with streptomycin or *p*-aminosalicylic acid.

METHODS AND MATERIALS

The full details of the method used have been described previously by Rees and Robson (1950), who also discuss the use of the method in assessing the activity of a drug. Corneal

* At present on the scientific staff of the Medical Research Council.

tuberculosis was produced by intracorneal injections of about 1,000 viable organisms of a bovine strain of *Mycobact. tuberculosis*.

In the present investigation all treatments were maintained for a standard period of twenty-eight days. Streptomycin was given subcutaneously, and the total daily dose of 4 mg. was injected in two halves, i.e., 2 mg. in 0.2 ml. sterile distilled water, morning and evening. On Saturdays and Sundays only a single dose of 2 mg. was given in the morning. Treatment was started within a few hours of inoculation. *p*-Aminosalicylic acid (2 per cent) and thiosemicarbazone (0.085 per cent) were given thoroughly mixed into the powdered diet and treatment was started some twenty-four hours before inoculation.

Results

Thiosemicarbazones.—A number of thiosemicarbazones were investigated (Bavin, Rees, Robson, Seiler, Seymour, and Suddaby, 1950), including 4-acetamidobenzaldehyde thiosemicarbazone (TB1/698) and 4-ethylsulphonylbenzaldehyde thiosemicarbazone (TB3/1374). All those which showed activity *in vivo* behaved in a very similar manner, so that only the results obtained with the ethylsulphonylbenzaldehyde compound need be described here. Preliminary toxicity experiments, in which these compounds were mixed in the diet, showed that mice tolerated approximately 5 to 10 mg. per day. With these doses there is an initial loss of weight due to loss of appetite; this confirms the observation of Hoggarth, Martin, Storey, and Young (1949). With the lower dose the mice recover after a week, but with the higher dose a number of mice ultimately die. The 5-mg. dose level was used in all the present experiments, and in fact gave chemotherapeutic results as good as the higher dose.

The following results were obtained in a typical experiment: seven mice in the untreated control group all developed tuberculous corneal lesions between the 11th and 14th days after inoculation, and these rapidly increased in size (Fig. 1A);

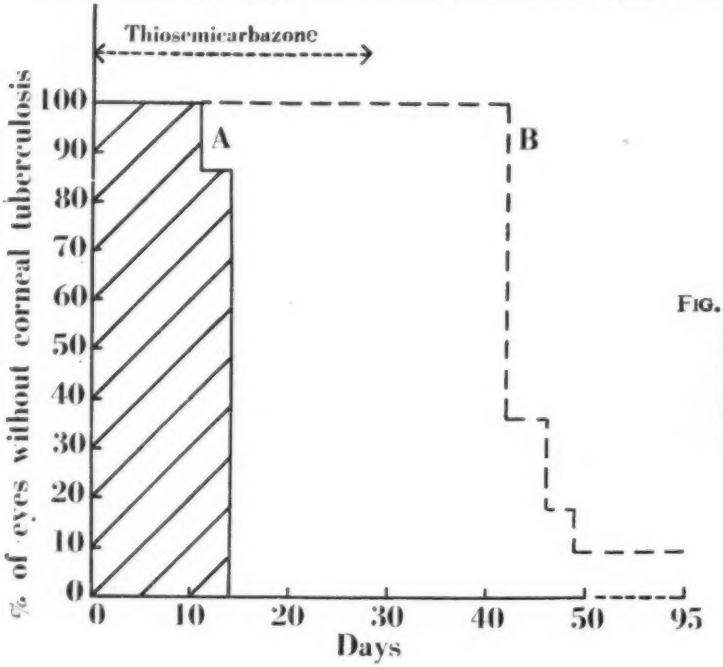

FIG. 1.—To show the effect of thiosemicarbazone on the development of corneal tuberculosis. (A) Control group. (B) Mice treated with 4-ethylsulphonylbenzaldehyde thiosemicarbazone.

eleven mice in the group treated with thiosemicarbazone remained free from corneal tuberculosis throughout the period of treatment; seven of these developed lesions on the 42nd day (i.e., 14 days after cessation of treatment), two on the 46th day, and one on the 49th day after inoculation. The one remaining mouse had a clear cornea when the experiment was terminated on the 95th day (Fig. 1B). The incubation period was markedly prolonged in all the treated mice, but the lesions, once they had appeared, rapidly increased in size and in no way differed from those seen in the untreated control animals.

Thiosemicarbazone and p*-aminosalicylic acid.*—In this experiment seven untreated control mice all developed corneal tuberculosis between the 11th and 14th days after inoculation. Of the six mice on *p*-aminosalicylic acid, five developed lesions between the 18th and 24th days, while still under treatment, and the remaining mouse on the 32nd day (Fig. 2A). All these lesions were active in type. The six

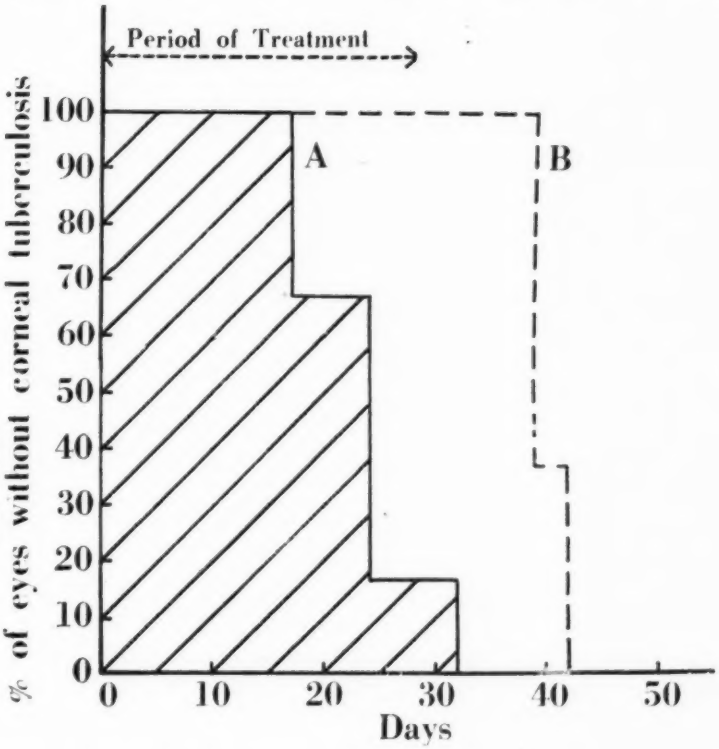

FIG. 2.—To show the effect of *p*-aminosalicylic acid alone (A) and in combination with thiosemicarbazone (B) on the development of corneal tuberculosis.

mice on thiosemicarbazone remained free from corneal tuberculosis throughout the 28-day period of treatment, but all developed active lesions between the 39th and 50th days. Eight mice were given both *p*-aminosalicylic acid and thiosemicarbazone; they remained free from corneal tuberculosis during the period of treatment, but all developed active corneal lesions between the 39th and 42nd days (Fig. 2B.)

Thiosemicarbazone and streptomycin.—In one such experiment there were eight untreated control mice, of which seven developed corneal lesions on the 11th day,

and the remaining one on the 18th day after inoculation; all were typical acute lesions. There were eleven mice on streptomycin, four of which developed corneal lesions between the 18th and 25th days while still under treatment; six of the remaining seven developed lesions between the 32nd and 46th days, and the remaining mouse was clear when the experiment was terminated 168 days after inoculation (Fig. 3A). However, the lesions in these animals on streptomycin were not all typical and acute. In six of the mice they remained minimal in size and in only four did the lesion become acute and similar to those seen in the untreated control series. There were eight mice on thiosemicarbazone, and they all remained free from corneal

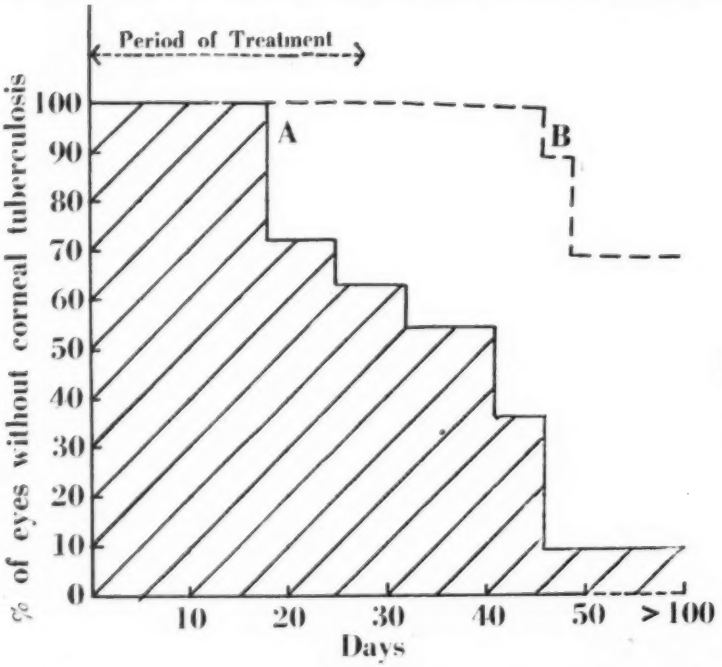

FIG. 3.—To show the effect of streptomycin alone (A) and in combination with thiosemicarbazone (B) on the development of corneal tuberculosis.

tuberculosis throughout the period of treatment; six of them developed lesions between the 42nd and 46th days and the remaining two on the 63rd and 100th day after inoculation respectively. All were typical acute lesions. On the other hand, in the group of ten mice on combined treatment only three developed corneal tuberculosis; one lesion appeared on the 46th day and remained minimal in size, and the other two appeared on the 49th day and developed into acute lesions. The remaining seven mice were still free from corneal tuberculosis when the experiment was terminated on the 168th day (Fig. 3B).

DISCUSSION

The results show that the mouse corneal method has again proved satisfactory for screening compounds for antituberculous activity, demonstrating that thiosemicarbazones possess marked chemotherapeutic activity. Indeed, additional information has been obtained which may help to throw some light on the mode of action of various drugs active in tuberculosis.

Two main criteria can be used in assessing the activity of a drug given for the standard period of 28 days: (1) the incubation period, which may fall within the 12–14 days range usually found in untreated animals or be prolonged; and (2) the type and development of lesions appearing after the incubation period. By recording these effects it has been found that each drug, or group of drugs, produces a characteristic and reproducible pattern of response. For example, with the thiosemicarbazones, and this applies not only to the 4-ethylsulphonylbenzaldehyde compound tested here but to all the active derivatives we have so far tested (Bavin *et al.*, 1950), no tuberculous lesions appear in the cornea during the 28-day period of treatment; this in itself is a characteristic which distinguishes this group of drugs from any other so far studied, but even more interesting is the observation that, despite this prolongation of the incubation period beyond the period of treatment, the eyes eventually develop tuberculous lesions at a characteristic period. The resistance of almost every eye breaks down between the 40th and 45th day, and a progressive lesion then appears which is in every way as severe as those seen in the untreated control group. Thus fifty-two mice have so far been treated for the standard 28-day period with an active thiosemicarbazone, and forty-eight of these have developed lesions with a mean incubation period of 43.8 days. The mean incubation period for the controls to these experiments was 13.3 days. Hence the mice treated with thiosemicarbazone developed lesions 15.8 days after the cessation of treatment, a period which, allowing for the elimination of the drug present in the mice on the last day of treatment, corresponds almost exactly with the mean incubation period of their controls. These results suggest that, under the conditions of these experiments, thiosemicarbazones exert essentially a bacteriostatic action, and that once the treatment has come to an end tubercle bacilli are still present in the cornea of most of the mice in sufficient number and with a sufficient degree of virulence to produce a corneal lesion with an incubation period and a subsequent rate of progress identical with that seen in untreated control mice.

The two experiments in which ethylsulphonylbenzaldehyde thiosemicarbazone was used in combination with another drug gave clear-cut results; this cannot always be achieved with other methods. Indeed, in experiments with potent chemotherapeutic substances it is often difficult to detect any advantage from combined therapy, and it may be necessary to reduce the doses considerably before it can be said with confidence that the result is not attributable to either drug used; this does not apply to the mouse corneal test, at least not with the drugs tested so far. Although streptomycin, thiosemicarbazones, and *p*-aminosalicylic acid all prolong the incubation period, there is subsequently a high incidence of lesions; even with streptomycin 50–90 per cent of the eyes develop lesions (though about half of them remain minimal), and with *p*-aminosalicylic acid and thiosemicarbazones more than 90 per cent succumb to the infection and develop progressive lesions. There remains therefore considerable scope for demonstrating any therapeutic advantage which a combined therapy may possess.

In the experiment in which a combination of *p*-aminosalicylic acid and thiosemicarbazone was used there was no evidence of an additive effect: the group of mice which received the combined treatment was no better than that receiving thiosemicarbazone only. This suggests, incidentally, that the mechanism of action

of *p*-aminosalicylic acid and thiosemicarbazones is rather similar, the latter group of drugs being more effective than *p*-aminosalicylic acid.

On the other hand the results in the mice treated with a combination of thiosemicarbazone and streptomycin were unequivocally better than those in the animals treated with either drug alone. In the experiment described here seven out of ten mice on combined therapy were free from corneal tuberculosis when the experiment was terminated 140 days after the end of treatment. This represents an additive effect even more striking than that obtained by the same method with a combination of streptomycin and *p*-aminosalicylic acid (Rees and Robson, 1950).

Summary

1. Corneal tuberculosis, produced by an intracorneal injection of about 1,000 viable organisms of a bovine strain of *Mycobact. tuberculosis*, was used to assess the chemotherapeutic activity of 4-ethylsulphonylbenzaldehyde thiosemicarbazone (TB3/1374) given alone and in combination with *p*-aminosalicylic acid or streptomycin.

2. TB3 and other active thiosemicarbazones tested showed marked antituberculous activity, producing a prolongation of the incubation period, but over 90 per cent of eyes eventually developed active tuberculosis. The results suggest that, under the conditions of this test, the action of thiosemicarbazones is essentially bacteriostatic.

3. A combination of *p*-aminosalicylic acid and thiosemicarbazone showed no advantage over thiosemicarbazone alone.

4. A combination of streptomycin and thiosemicarbazone showed a definite additive effect, greater than that produced under the same experimental conditions by a combination of streptomycin and *p*-aminosalicylic acid.

We are grateful to the W. H. Ross Foundation (Scotland) for the Prevention of Blindness, which defrayed some of the expenses. The streptomycin was kindly supplied by the Tuberculosis Study Section of the U.S. Public Health Service (through Dr. Schmehl), the *p*-aminosalicylic acid by Herts Pharmaceuticals, Ltd. (through Mr. Seymour), and the thiosemicarbazone by Imperial Chemical Industries, Ltd. (through Dr. Martin).

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THE ENHANCEMENT OF THE EFFECT OF ORAL PENICILLIN IN LABORATORY ANIMALS BY THE USE OF OIL CONTAINING ALUMINIUM RESINATE

BY

Y. C. HSU AND G. A. H. BUTTLE

From the Department of Pharmacology, School of Pharmacy, University of London

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Libby (1945) and many other workers (McDermott, Bunn, Benoit, Dubois, and Haynes, 1945; Perlstein, Kluener, and Liebmann, 1945; Finland, Meads, and Dry, 1945; Rake and Richardson, 1946) employed vegetable oils alone or with other substances as suspending media for penicillin given orally. As oils are not miscible with water, they might well form an ideal protection for the penicillin in the stomach. As the oil is absorbed in the small intestines, the site where the maximum absorption of oral penicillin occurs, penicillin might be carried there by the oil and both might be absorbed at the same time. Thomas, Lyons, Romansky, Rein, and Kitchen (1948) reported the use of a water-repelling substance, aluminium stearate, with oil in delaying the absorption of intramuscular penicillin. It was therefore thought that a water-repelling agent might increase the protective effect of the oil on oral penicillin.

Among many water-repellents tested aluminium resinate was found to be the most effective in mice, rats, guinea-pigs, and rabbits. The present paper gives an account of experimental results in the course of an investigation of this effect of aluminium resinate.

METHODS

Blood penicillin levels.—Five species of laboratory animals, viz., mouse, rat, guinea-pig, rabbit, and cat, were used. Doses were given by means of a stomach tube. The " oral dose " referred to in this paper was given by this method. Blood samples were withdrawn at different intervals by heart puncture in all species except the rabbit, in which blood samples were obtained from the ear veins. In the mouse, 0.3 ml. of blood was withdrawn from each of three animals and the specimens were then pooled. In the rat, successive blood samples for a test were obtained from each of the same three animals. In a similar manner, in the guinea-pig, blood samples were obtained from each of the same two animals and in the cat or rabbit from the same one animal. The potencies of penicillin in different blood samples were compared by the Agar-cup plate method.

Infection test.—Mice (18–22 g.) of either sex were used. Tests were designed to compare the degree of protection given by various penicillin preparations against a haemolytic streptococcal infection. The mice were infected intraperitoneally by an inoculum of 0.2 ml. of a 1: 10,000 dilution of a 6-hour blood-broth culture of the Richards strain of *Streptococcus haemolyticus*. The doses of the various penicillin preparations were all of a potency equivalent to 10 units of penicillin. Other methods will be described later.

Results

Effect of oil on oral penicillin in mice

In order to confirm Libby's theory of the beneficial action of an absorbable oil as a protective agent, the effects of various oils were compared. It was found (Table I) that if penicillin was given orally together with an absorbable oil such as cod-liver oil, olive oil, or arachis oil, an increased blood level was obtained; if on the other hand it was given with the non-absorbable liquid paraffin the levels were no better than those in the control animals.

TABLE I

BLOOD PENICILLIN LEVELS IN GROUPS OF MICE AFTER ORAL PENICILLIN IN VARIOUS OILS

9 mice in each group. Penicillin levels estimated by Agar-cup plate method. D = diameter of inhibition zone in mm. U./ml. = units of penicillin per ml. of blood

Oral dose: (500 u. calcium penicillin in 0.2 ml. oil)	Blood penicillin level at various times after dose				
	1 hr.		2 hr.		4 hr.
	D	U./ml.	D	U./ml.	
Cod-liver oil ..	16	0.48	17	0.50	0
Olive oil	17	0.53	14	0.39	0
Arachis oil	18	0.58	15	0.44	0
Liquid paraffin ..	12	0.32	0	0	0
Control (water) ..	13	0.35	0	0	0

Arachis oil (later referred to as oil) was used throughout the experiments to be described as a medium in which calcium penicillin was to be suspended; 0.2 ml. of this oil was used to suspend 500 units of calcium penicillin. This preparation was found to give as good blood levels in mice as an aqueous preparation containing 50 mg. sodium citrate and 500 units of sodium penicillin (Table II).

TABLE II

BLOOD PENICILLIN LEVELS IN GROUPS OF MICE AFTER ORAL PENICILLIN IN VARIOUS AMOUNTS OF ARACHIS OIL

9 mice in each group. Symbols as in Table I

Oral dose	Blood penicillin level after				
	1 hr.		2 hr.		4 hr.
500 u. Ca penicillin in :	D	U./ml.	D	U./ml.	
0.1 ml. arachis oil ..	15	0.44	0	0	0
0.2 ml. arachis oil ..	20	0.70	14	0.39	0
0.5 ml. arachis oil ..	22	0.85	14	0.39	0
500 u. Na penicillin with 50 mg. Na citrate ..	18	0.58	15	0.44	0

Aluminium resinate

Aluminium resinate, derived from colophony resin, is widely used in paint and paper industries. It was tested because of its water-repelling property.

(*a*) *Acute toxicity of aluminium resinate in mice.*—Doses of 5, 10, 20, 50, 100, 200, and 400 mg. of aluminium resinate per 20 g. mouse were given by mouth to

different groups of mice. Doses of 5, 10, or 20 mg. per 20 g. mouse were non-toxic and the LD50 by mouth was found to be 8.75 g. per kg.

(*b*) *The effect of aluminium resinate in oil on oral penicillin in mice.*—20 mg. aluminium resinate in 0.2 ml. oil per 20 g. mouse was found to enhance the blood penicillin level after oral penicillin. A number of tests were carried out in mice in which the blood levels after the administration of various preparations of penicillin were compared. These included the following:

1. Al resinate (20 mg.) and calcium penicillin (500 units) in 0.2 ml. oil given orally.
2. Procaine penicillin (500 units) in 0.1 ml. oil containing 2 per cent Al stearate given intramuscularly.
3. Sodium citrate (50 mg.) and sodium penicillin (500 units) in 0.2 ml water given orally.
4. Calcium penicillin (500 units) in 0.2 ml. oil given orally.

The results are shown in Table III.

At equivalent dose levels the mice receiving oral penicillin with aluminium resinate in oil gave even higher blood penicillin levels than those receiving intramuscular procaine-penicillin. Oral penicillin with sodium citrate or oil alone both gave lower figures (Fig. 1).

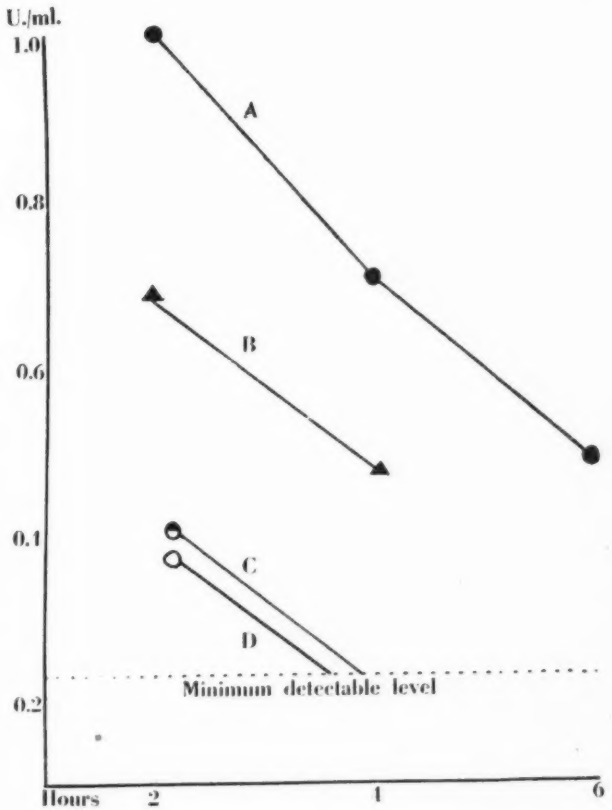


FIG. 1.—A comparison of the blood penicillin levels in mice after (A) oral penicillin (500 u.) in 0.2 ml. oil with 20 mg. Al resinate; (B) intramuscular procaine-penicillin (500 u.); (C) oral penicillin (500 u.) with 50 mg. Na citrate; and (D) oral penicillin (500 u.) in 0.2 ml. oil. Doses are quoted per 20 g. mouse.

TABLE III

BLOOD PENICILLIN LEVELS IN GROUPS OF MICE AFTER ORAL PENICILLIN PREPARATIONS AND INTRAMUSCULAR PROCAINE PENICILLIN

9 mice in each group. Symbols as in Table I, except that a dash means zero level

Dose	Group No.	Blood penicillin level after					
		2 hr.		4 hr.		6 hr.	
		D	U./ml.	D	U./ml.	D	U./ml.
Oral: Ca penicillin, 500 u. Al resinate, 20 mg. Oil, 0.2 ml.	1	26	1.25	20	0.70	13	0.35
	2	26	1.25	23	0.94	14	0.39
	3	26	1.25	17	0.53	19	0.63
	4	20	0.70	19	0.63	17	0.53
	5	26	1.25	13	0.35	18	0.58
	6	22	0.85	21	0.77	19	0.63
	7	20	0.70	22	0.85	13	0.35
	8	20	0.70	23	0.94	17	0.53
	Mean		1.00		0.71		0.50
I.M.: Procaine penicillin, 500 u. Oil, 0.1 ml. (containing 2% Al stearate)	1	22	0.85	17	0.53	—	—
	2	21	0.77	15	0.44	—	—
	3	20	0.70	17	0.53	—	—
	4	17	0.53	14	0.39	—	—
	5	18	0.58	18	0.58	14	0.39
	6	19	0.63	18	0.58	—	—
	7	19	0.63	14	0.39	—	—
	8	21	0.77	14	0.39	—	—
	Mean		0.68		0.48		
Oral: Ca penicillin, 500 u. Na citrate, 50 mg. Water, 0.2 ml.	1	17	0.53	—	—	—	—
	2	12	0.32	—	—	—	—
	3	16	0.48	—	—	—	—
	4	15	0.44	—	—	—	—
	5	14	0.39	—	—	—	—
	6	12	0.32	—	—	—	—
	7	13	0.35	—	—	—	—
	Mean		0.40		—		—
Oral: Ca penicillin, 500 u. Oil, 0.2 ml.	1	11	0.29	—	—	—	—
	2	12	0.32	—	—	—	—
	3	12	0.32	—	—	—	—
	4	15	0.44	12	0.32	—	—
	5	13	0.35	—	—	—	—
	6	15	0.44	—	—	—	—
	7	17	0.53	—	—	—	—
	8	12	0.32	—	—	—	—
	9	12	0.32	—	—	—	—
	Mean		0.38		—		—

(*c*) *The effect of aluminium resinate on oral penicillin in the rat, guinea-pig, rabbit, and cat.*—In a series of experiments (Table IV) it was found that the rat and guinea-pig responded to aluminium resinate in the same way as the mouse; the rabbit did not give such consistent results, but five out of six cross-over experiments gave a positive result; the cats used for cross-over experiments showed no effect.

(*d*) *Oral aluminium resinate and subcutaneous penicillin.*—Many substances, e.g., caronamide (Crosson, Boger, Shaw, and Miller, 1947) and benzoic acid (Waldo and Lee, 1948), enhance penicillin blood levels by delaying the excretion of penicillin

TABLE IV

BLOOD PENICILLIN LEVELS IN RATS AND GUINEA-PIGS AFTER ORAL PENICILLIN IN OIL WITH AL RESINATE

Species and body weight	Dose: g. or u. per kg. body weight	No. of test	Blood penicillin level (as inhibition zone in mm.) after			
			1 hr.	2 hr.	4 hr.	6 hr.
Rats: 150±10 g. 3 in each group	Test: Al resinate ,1 g.	(1)		15	14	13
	Ca penicillin, 25,000 u.	(2)		18	18	14
	Oil, 5 ml.	(3)		16	13	—
	Control: Ca penicillin, 25,000 u.	(1)		12	—	—
	Oil, 5 ml.	(2)		11	—	—
		(3)		13	—	—
Guinea-pigs: (1) 250 g. 260 g. (2) 270 g. 280 g.	Test: Al resinate, 1 g. Ca penicillin, 25,000 u. Oil, 5 ml.	(1) (2)	22 18	18 17	13 11	
(1) 220 g. 280 g. (2) 235 g. 265 g.	Control: Ca penicillin, 25,000 u. Oil, 5 ml.	(1) (2)	14 14	11 13	— —	
Rabbits: No. 1 wt. 2.05	Test: Al resinate, 1 g. Ca penicillin, 50,000 u. Oil, 5 ml.	(1)		15	—	—
2 2.20		(2)		19	15	10
3 1.37		(3)		14	13	—
4 1.43		(4)		13	—	—
5 2.10		(5)		13	—	—
6 2.10		(6)		11	—	—
2 2.20	Control: Ca penicillin, 50,000 u. Oil, 5 ml.	(1)		—	—	—
1 2.05		(2)		—	—	—
4 1.43		(3)		—	—	—
3 1.37		(4)		—	—	—
6 2.10		(5)		19	15	—
5 2.10		(6)		17	—	—
Cats: No. 1 wt. (g.) 650	Test: Al resinate, 1 g. Ca penicillin, 25,000 u. Oil, 5 ml.	(1)		15	16	15
2 600		(2)		22	21	20
1 650		(3)		19	18	14
2 600	Control: Ca penicillin, 25,000 u. Oil, 5 ml.	(1)		22	17	15
1 650		(2)		16	—	16
2 600		(3)		20	17	16

through competition with it in tubular excretion. Tests were made to try to find any indication of this sort of action by aluminium resinate. Four groups of three mice, A, B, C, and D, were treated as follows: (A) oral penicillin in oil with aluminium resinate; (B) subcutaneous penicillin in saline and oral aluminium resinate in oil;

(C) oral penicillin in oil; (D) subcutaneous penicillin in saline. Blood levels were estimated as usual.

Fig. 2 shows the result of this test. Comparison of curves B and D will show that the blood level after subcutaneous penicillin was enhanced by oral aluminium resinate; this suggests that aluminium resinate may possess a " caronamide-like " action. Comparison of curves A and B will reveal that in these two groups both with oral aluminium resinate, the group with oral penicillin gave consistent and longer blood penicillin levels, whereas the group with subcutaneous penicillin gave higher blood penicillin levels at the start which decreased rapidly afterwards. From a comparison of the two pairs of curves A, C and B, D, it appears that aluminium resinate increased the effect of penicillin given orally more than it increased the effect of penicillin given subcutaneously.

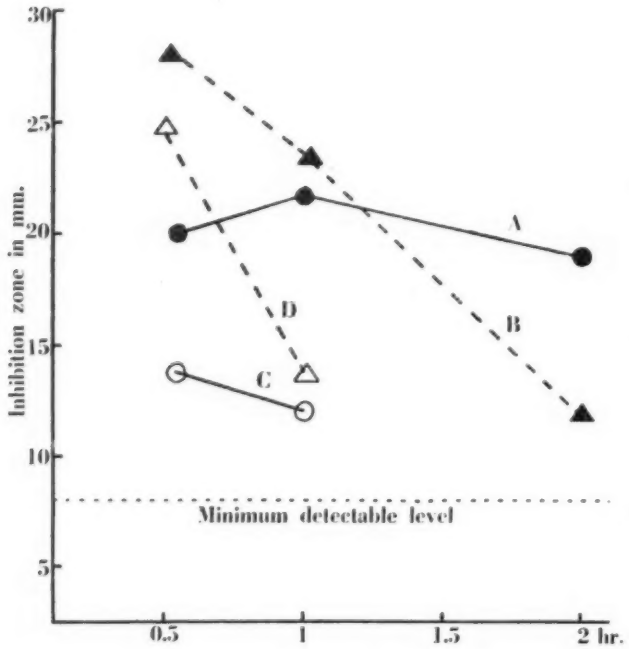


FIG. 2.—A comparison of blood penicillin levels in mice after (A) oral Ca penicillin (250 u). in 0.2 ml. oil with 10 mg. Al resinate; (B) subcutaneous Ca penicillin (250 u.) in 0.1 ml. saline and oral Al resinate (10 mg. in 0.2 ml. oil); (C) oral Ca penicillin (250 u.) in 0.2 ml. oil; and (D) subcutaneous Ca penicillin (250 u.) in 0.1 ml. saline. Doses per 20 g. mouse. Experiment repeated 3 times so that each point represents the average value for nine mice.

(*e*) *Protection of mice against a haemolytic streptococcal infection by oral penicillin with aluminium resinate in oil.*—A series of tests were performed to see whether the enhancement in blood penicillin levels caused by the use of aluminium resinate would induce a higher percentage of survivors in mice infected with haemolytic streptococci.

The effect of treatment with oral penicillin and aluminium resinate was compared with the effects of treatments with intramuscular procaine-penicillin and with oral penicillin and sodium citrate. Three groups, each of 26 mice, were treated immediately after infection with one of the following agents:

A—Oral calcium penicillin (10 units) in 0.2 ml. oil containing aluminium resinate (20 mg.).

B—Intramuscular procaine-penicillin (10 units) in 0.1 ml. oil containing 2 per cent aluminium stearate.

C—Oral sodium penicillin (10 units) with sodium citrate (50 mg.) in 0.2 ml. water.

Three other groups of mice were included as various controls:

D—Oral calcium penicillin (10 units) in 0.2 ml. oil (30 mice).

E—Oral aluminium resinate (20 mg.) in 0.2 ml. oil (10 mice).

F—Untreated (20 mice).

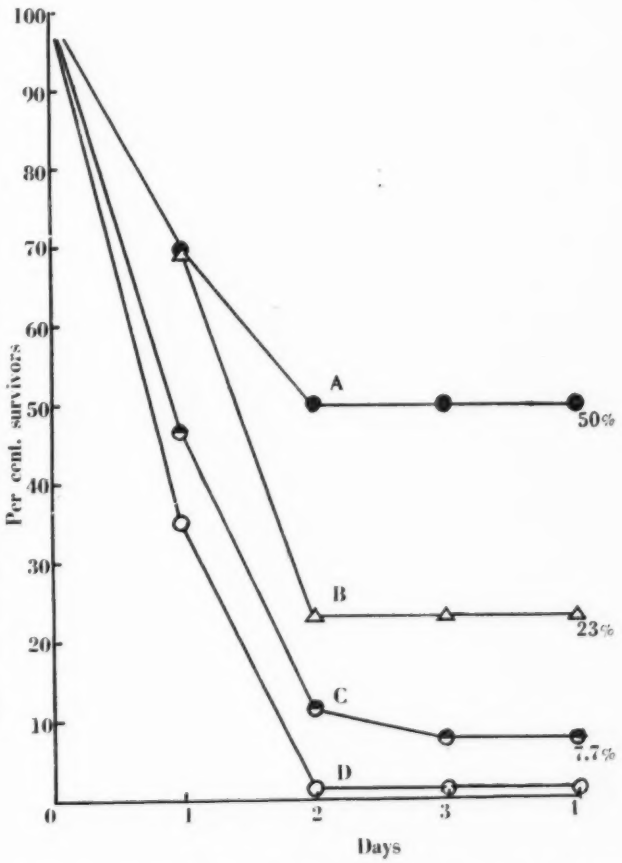


FIG. 3.—Protection of mice against haemolytic streptococcal infection. Survival curves: (A) after oral penicillin (10 u.) in 0.2 ml. oil with 20 mg. Al resinate; (B) after intramuscular procaine-penicillin (10 u.); (C) after oral penicillin (10 u.) with 50 mg. Na citrate; and (D) after oral penicillin (10 u.) in 0.2 ml. oil. Doses per 20 g. mouse. All infected mice, untreated or given an oral dose of aluminium resinate (20 mg.), died on the first day of infection. All infected mice given oral penicillin in oil alone (D) dead within two days.

Fig. 3 gives the percentages of survivors each day after the infection. It will be noticed that oral aluminium resinate with penicillin in oil gave the highest degree of protection (50 per cent), that intramuscular procaine-penicillin was the second best (23 per cent), and that aqueous penicillin with sodium citrate gave the lowest figure (7.7 per cent). In the control groups, all the mice treated with oral penicillin in oil died within three days and those untreated or receiving oral aluminium resinate in oil died within twenty-four hours after infection.

(*f*) *Effect of aluminium resinate on the kidneys.*—Two groups of mice (6 in each group) were fed with normal diet and with diet containing 2 per cent of aluminium

resinate respectively for 14 days. Two mice from each group were killed by use of ether. The kidneys were then dissected out and sectioned. Microscopical examination showed no obvious difference between the kidneys of the mice receiving normal diet and those of the mice receiving diet containing Al resinate. No sign of epithelial degeneration in the tubules was observed.

Three groups of rats (5 in each group, approximately 200 g. each) were fed respectively with normal diet and with diet containing 2 and 5 per cent of Al resinate for 13 days. On the 14th day, 0.1 g. albumin was added to every 10 g. of the various kinds of diet. The next day, a urine sample from each group was collected. The urine was tested for the presence of albumin both with sulphosalicylic acid reagent and with glacial acetic acid. On addition of the reagent, slight cloudiness was observed in the urine passed by the rats with normal diet. Voluminous precipitates were obtained in the samples of urine passed by rats with diet containing Al resinate. Al resinate therefore increased albuminuria in rats.

(*g*) *Test for any* in vitro *effect of aluminium resinate*.—It was found that the addition of 20 mg. aluminium resinate to 4.5 ml. nutrient broth had no influence on the growth of either *Staphylococcus aureus* or *Escherichia coli*. The addition of this quantity of resinate did not influence the point at which penicillin would inhibit the growth of either of these organisms.

(*h*) *Estimation of urinary penicillin in animals after an oral dose of penicillin with Al resinate*.—As an increase of urinary excretion of penicillin is a good indication of increased absorption or of increased stability of penicillin, an estimation of the urinary penicillin was made in rats and rabbits. Rats, 4 in each group, were kept in metabolism cages the bottom parts of which were waxed to prevent the direct contact of urinary penicillin with metals. The urine was collected at intervals for five hours after the dose. The collections were immediately Seitz-filtered and then made up to a definite volume. The potency of penicillin was estimated by the serial dilution method. It was found that the group receiving oral penicillin with Al resinate excreted twice as much penicillin as the control group (Table V).

TABLE V

THE URINARY PENICILLIN IN RATS AFTER ORAL PENICILLIN IN OIL WITH AND WITHOUT AL RESINATE

4 rats in each group. Weight 200±10 g.

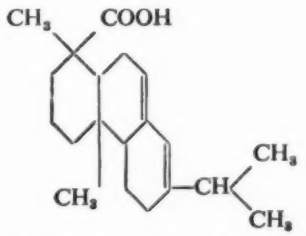
Dose (per rat)	Time (hr.) of collection of urine after dose	Volume (ml.) of urine collected	Total urinary penicillin (u.)	Percentage recovery
Control: Ca penicillin, 3,000 u. Oil, 0.5 ml.	2	14	200	5
	5	8	400	
Test: Al resinate, 0.2 g. Ca penicillin, 3,000 u. Oil, 0.5 ml.	2	17	400	10
	5	12	800	

Urine was also collected from two groups of rabbits which received oral doses of penicillin in oil and penicillin in oil with aluminium resinate respectively. No

significant difference was found in the urinary recoveries between the control and the test groups.

(*i*) *Abietic acid derivatives and copals.*—In view of the activity of Al resinate in mice, the study was extended to allied compounds. Many metallic resinates are available commercially, but as ions of most heavy metals are toxic only calcium resinate was tried. It was found to be as effective as Al resinate both in enhancing blood penicillin levels in mice and in protecting mice against a haemolytic streptococcal infection.

The substance colophony resin, from which Al resinate is prepared, contains about 85 per cent resinic acid which is chiefly abietic acid. The structural formula of abietic acid is shown as follows:



Tests on blood penicillin levels in mice showed that this compound was effective. The abietic acid molecule seemed therefore to be responsible for the activity of Al and Ca resinates. To follow up the research, four classes of abietic acid derivatives were examined. These were as follows:

(1) *Salt.*—Al abietate; a salt (composition unknown) prepared by treating alum with abietic acid.

(2) *Esters.*—Methyl abietate, glyceryl abietate, and pentaerythritol abietate.

(3) *Alcohol.*—Hydroabietyl alcohol; this is a partially hydrogenated abietic acid with the carboxyl group reduced to a primary alcohol group.

(4) *Derivatives with changed cyclic structures*

(*a*) Hydrogenated: " Stayblite "; this is a hydrogenated abietic acid in which one of the two double bonds has been saturated by catalytic hydrogenation.

(*b*) Dehydrogenated: " Resin 731 "; this is a partially dehydrogenated abietic acid so that an additional double bond has been introduced.

(*c*) Polymerized: " Polypale resin "; this is the dimer of abietic acid.

(*d*) Addition compound: " Fumaric modified rosin " (lewisol 40); this is the simple combination of fumaric acid and abietic acid so that it is, in effect, a tricarboxylic acid.

These compounds were tested for their activity in enhancing blood penicillin levels in mice after oral penicillin. In this respect, Al abietate was found to be as effective as Al resinate. Three other derivatives, Stayblite, Resin 731, and Polypale resin, were also found effective but more toxic than Al resinate.

It is interesting to note that the metallic salt and those derivatives in which the carboxyl group is free were active with the sole exception of " Fumaric modified rosin," and that the esters and the alcohol derived from abietic acid were inactive.

Copals are of diverse botanical origins. As they are strongly water repelling, five samples of copal were tested. These were as follows:

Congo copal; Kauri (candle bush); Zanzibar (goose skin); Manila (half-hard); and fused Congo copal.

The first four resins are natural products composed of mixtures of acids and non-acids. Their composition has not been established with any certainty, but the acids contained therein, while bearing some structural relationship to abietic acid, are polycarboxylic and mainly of higher molecular weight. Fused Congo copal is Congo copal which has been destructively heated to render it soluble in glyceride oils.

Tests were made on each of these compounds. It was found that there were enhanced blood penicillin levels in mice after an oral dose of penicillin and fused Congo copal in arachis oil.

As fused Congo copal is a mixture, different organic solvent fractions were tested. It was found that the ether fraction enhanced oral penicillin in mice to a greater extent than any of the other fractions, namely, the petroleum ether fraction, the ether-ethanol fraction, and the ether-methanol fraction.

Discussion

The use of absorbable oils as protective agents for oral penicillin was supported by the experiment in this paper in which oral penicillin given in liquid paraffin, a non-absorbable oil, did not give as good a result as when it was given in arachis oil, olive oil, or cod-liver oil. It might be true, as Rake and Richardson (1946) pointed out, that the salts of penicillin being highly water-soluble would not remain in the oil phase for more than a short period of time after mixing with gastric contents. The addition of aluminium resinate, a water-repellent, to the oil might be able to keep penicillin in the oil phase so that the oil could carry it to the site of its absorption. The water-repelling property of aluminium resinate is abolished in an alkaline medium; it is probable that in the small intestine owing to the relatively alkaline *p*H there is not sufficient water-repelling action to interfere with absorption.

Aluminium resinate in oil not only induces a high blood level in mice after oral penicillin but also enhances the protective effect of oral penicillin in mice against a haemolytic streptococcal infection. The mechanism of action of aluminium resinate is not fully known. Orally administered aluminium resinate also enhanced the effect of subcutaneous penicillin in mice, and this might suggest that as with caronamide this substance caused a delayed renal clearance of penicillin. Experiments on the urinary output of penicillin given with the resinate and oil in rats (but not in rabbits) showed that the total urinary penicillin was doubled; this suggests that there was increased absorption or increased stability rather than delayed excretion. That the principal action was due to increased absorption of oral penicillin was also suggested by the following experiments: (*a*) aluminium resinate increased the effect of oral penicillin more than it increased the effect of subcutaneous penicillin, and (*b*) treatment of a haemolytic streptococcal infection in mice with oral penicillin and aluminium resinate gave a much higher percentage of protection than treatment with oral penicillin and a dose of caronamide of the same weight as the aluminium resinate. (Note: the molecular weight of Al resinate calculated as Al abietate is 328.4 and that of caronamide 291.)

The activity of aluminium resinate in mice may be threefold; firstly, it may increase the protective effect of oil on penicillin in the stomach by acting as a water-repelling substance; secondly, it may promote absorption of penicillin in the intestine; and thirdly, it may delay excretion of penicillin through the kidney. There is, so far, no evidence that aluminium resinate itself shows any antibacterial activity either *in vivo* or *in vitro*.

Species differences were seen. The resinate is effective in mice, rats, and guinea-pigs. In rabbits it gave inconsistent results, while in cats no enhancement was obtained.

Abietic acid derivatives and other resins

The investigation was extended to include the essential component of aluminium resinate, *abietic acid*, and some of its simple derivatives in order to correlate the molecular structure with the activity of enhancing oral penicillin in mice. That the activity and the toxicity of aluminium abietate are of the same order as that of aluminium resinate suggests that it is the abietic acid molecule which is essential.

Copals

In the study of copals, not all raw copals have been found to be of use, but fused Congo copal showed some activity which was, however, not as high as that of aluminium resinate. It is hoped that fractionation or degradation of fused Congo copal or better still the determination of the chemical identities of the constituents of this highly complex mixture might reveal the essential feature with regard to its activity.

Summary

1. Aluminium resinate, when given orally to mice with penicillin in oil, is effective in increasing the blood penicillin levels. This effect is also shown in rats, guinea-pigs, and rabbits, but not in cats.

2. Aluminium resinate increases the effect of oral penicillin in protecting mice infected with a strain of haemolytic streptococci, and in this respect oral penicillin with aluminium resinate was found to be somewhat more effective than an equivalent amount of procaine penicillin injected intramuscularly.

3. The experimental results suggest that this action of aluminium resinate is due to the absorption of penicillin being increased and to its excretion being delayed.

4. Several derivatives of abietic acid (the essential component of aluminium resinate) and various copals were tested. Although these are all water-repelling agents, only a few of them showed an activity in enhancing blood penicillin levels in mice after oral penicillin.

5. The acute toxicity of aluminium resinate to mice is low, the LD50 by mouth being 8.75 grammes per kilogram.

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We should like to acknowledge a grant from the Medical Research Council to one of us (G.A.H.B.).

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Note added in proof.—Since this paper was written Dr. B. N. Halpern has kindly informed us that he has been able to obtain enhanced blood levels of penicillin administered orally in dogs and in human patients by using aluminium resinate in arachis oil.

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*NOR*ADRENALINE AND ADRENALINE IN VESSELS OF THE RABBIT EAR IN RELATION TO THE ACTION OF AMINE OXIDASE

BY

J. H. BURN AND JUDITH ROBINSON

From the Department of Pharmacology, Oxford University

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There is much variation in the activity of *nor*adrenaline compared with that of adrenaline. Barger and Dale (1910) observed that, whereas in the beginning of an experiment the pressor action of *nor*adrenaline was greater than that of adrenaline, at the end it was often less than that of adrenaline. Burn and Hutcheon (1949) recorded a similar change in the relative contracting action on the spleen. Since the cause of this varying relation is difficult to determine in the intact animal, we have sought to investigate it first in a simpler preparation, namely, the vessels of the rabbit ear perfused with Locke's solution, in which comparisons have already been made by different workers. There is fair agreement about the facts; Luduena, Ananenko, Siegmund, and Miller (1949) found that the ratio between amounts of *l-nor*adrenaline and *l*-adrenaline which produced the same constriction was 2.5 to 1.5; Gaddum, Peart, and Vogt (1949) found the ratio was 3.0 to 1.0; Burn and Hutcheon (1949) found the ratio was 4.0 to 1.0. Thus occasionally *l-nor*adrenaline is as active as *l*-adrenaline, but usually it is found to be weaker.

Experiments with perfused rabbit ears

Change in ratio with duration of perfusion.—The first point we investigated was whether the ratio varied according to the length of time the ear was perfused. The central artery of the ear was cannulated at the base and the outflow was collected in a funnel which took it to a recorder of the kind described by Stephenson (1948). The perfusion was at room temperature, the composition of the Locke solution being NaCl 9.0 g., KCl 0.42 g., $CaCl_2$ 0.24 g., $NaHCO_3$ 0.5 g., dextrose 1.0 g., glass distilled water 1 litre. In the later experiments oxygen was bubbled through the solution beforehand, though no difference was observed. We used *l-nor*adrenaline which was very kindly given to us by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute. Solutions of *nor*adrenaline and adrenaline were prepared to contain 10 μg./ml., and were acidified. From these a dilution in the perfusion fluid was freshly prepared before each injection into the artery cannula.

We found that when the ear was freshly perfused the action of *nor*adrenaline was weak compared with that of adrenaline, so that the ratio N/A of amounts of *nor*adrenaline and adrenaline producing the same constriction was high. After the perfusion had been continued overnight adrenaline was usually found to be more active and *nor*adrenaline still more active, so that the ratio was nearer to unity. Fig. 1

shows the similar vasoconstrictor action of 0.02 μg. *l-nor*adrenaline and 0.005 μg. *l*-adrenaline during the early stage of the perfusion; the ratio N/A is therefore = 4. In the lower part of the figure observations made the next morning, the perfusion

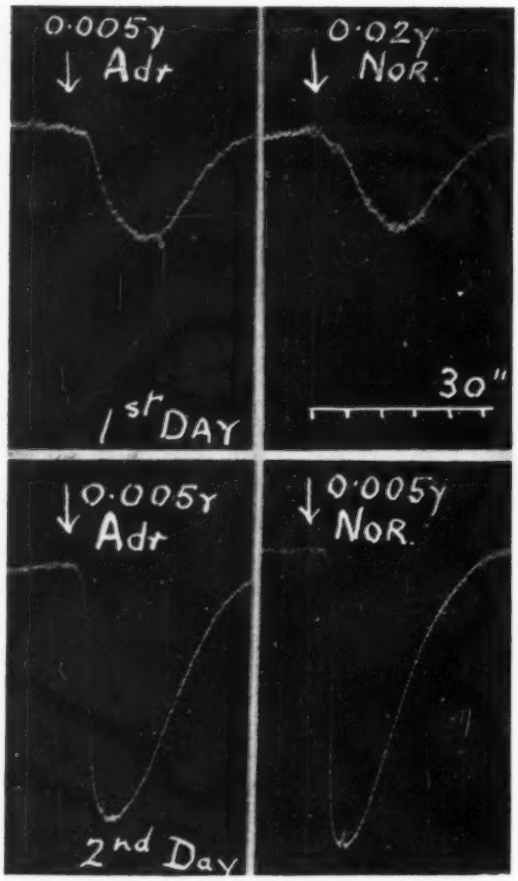

FIG. 1.—Records of the outflow from the vessels of the rabbit ear perfused with Locke's solution. A drop in the record indicates vasoconstriction. The two upper records were obtained during perfusion soon after the preparation had been set up. The two lower records were obtained about 24 hr. later. Note that on the first day 0.02 μg. *nor*adrenaline was needed to produce constriction equal to that of 0.005 μg. adrenaline. On the second day the vessels were more sensitive to adrenaline, and *nor*adrenaline was as active as adrenaline.

having continued overnight, showed that, while adrenaline was more active, *nor*adrenaline was much more active and the ratio N/A = 1. A series of 29 experiments of this kind was performed, and the results of the first 13 are given in detail in Table I, the mean ratio on the first day being 7.16 and on the second day 2.47. The mean ratio of all 29 experiments on the first day was 5.9 and on the second day 2.7, which are similar figures to those for the first 13 experiments. The actual amounts injected on the first day cannot in all cases be compared with the amounts injected on the second day, and these amounts give no precise guide to the change in sensitiveness. The point we wish to stress in the results is the change from the first day to the second in the relative amounts of *nor*adrenaline and adrenaline which have the same constrictor action.

The effect of ephedrine.—The presence of amine oxidase in the rabbit ear was first shown by Schapira (1945); it has recently been demonstrated by R. H. S. Thompson in the rabbit ear vessels (unpublished). It seemed possible that the fall in the N/A

TABLE I

AMOUNTS OF *l-nor*ADRENALINE AND *l*-ADRENALINE PRODUCING THE SAME VASOCONSTRICTION IN THE RABBIT EAR VESSELS DURING PERFUSION ON (*a*) THE FIRST DAY, (*b*) THE SECOND DAY

Exp.	1st day			2nd day		
	*Nor*adrenaline μg.	Adrenaline μg.	Ratio N/A	*Nor*adrenaline μg.	Adrenaline μg.	Ratio N/A
1	0.02	0.005	4	0.005	0.005	1
2	0.002	0.001	2	0.02	0.005	4
3	0.005	0.001	5	0.0125	0.005	2.5
4	0.1	0.01	10	0.02	0.015	1.3
5	0.06	0.01	6	0.15	0.05	3
6	0.01	0.001	10	0.15	0.05	3
7	0.02	0.0075	2.7	0.015	0.005	3
8	0.2	0.01	20	0.015	0.005	3
9	0.075	0.005	15	0.025	0.005	5
10	0.02	0.01	2	0.01	0.005	2
11	0.015	0.005	3	0.01	0.005	2
12	0.023	0.005	4.5	0.0075	0.005	1.5
13	0.02	0.005	4	0.015	0.015	1
Mean ratio of 13 experiments			7.16			2.47
Mean ,, ,, 29 ,, ...			5.9			2.7

ratio on the second day might be related to a diminution either in the amount or in the activity of the amine oxidase present. Burn and Hutcheon (1949) have already suggested that the inactivity of *nor*adrenaline on the normally innervated pupil and on the normally innervated nictitating membrane might be explained by the presence at the nerve ending of a high concentration of an enzyme which destroys *nor*adrenaline more easily than it destroys adrenaline.

To test the idea that the high N/A ratio on the first day is due to the activity of amine oxidase, we made use of the observations of Blaschko, Richter, and Schlossmann (1937) that ephedrine inhibits the action of amine oxidase. We carried out experiments in which the N/A ratio in the rabbit ear vessels was determined (*a*) when the preparation was fresh, (*b*) during perfusion with Locke's solution containing ephedrine hydrochloride, and (*c*) during a further period of perfusion with Locke's solution alone. If the high ratio for N/A in the fresh preparation was due to the more rapid destruction of *nor*adrenaline, then during perfusion with Locke containing ephedrine the enzyme responsible for the destruction might be inhibited, and the ratio N/A might fall to a value nearer unity. When the ephedrine was removed, the ratio might then rise again.

All experiments were completed within a few hours of setting up the preparation, so that the results could not be affected by oedema. Ephedrine hydrochloride was added to the Locke's solution in the amount of 10 μg. per ml., which was the concentration used by Gaddum and Kwiatkowski (1938) when they first demonstrated the increased sensitiveness of the vessels to adrenaline and to sympathetic stimulation in the presence of ephedrine. The details of the results in 7 experiments are given in Table II, though 13 experiments were made in all, and the mean ratios N/A for all 13 experiments are also given. Satisfactory agreement was obtained between the mean ratio 5.5 in Table II obtained when the preparation was first perfused and the similar ratio 5.9 obtained

TABLE II

CHANGES IN THE RATIO OF AMOUNTS OF *nor*ADRENALINE AND ADRENALINE PRODUCING THE SAME CONSTRICTION IN RABBIT EAR VESSELS BEFORE, DURING, AND AFTER THE ADDITION OF EPHEDRINE TO THE PERFUSING FLUID

Exp.	At first			During ephedrine perfusion			After ephedrine perfusion		
	Nor-adrenaline μg.	Adrenaline μg.	N/A	*Nor*-adrenaline μg.	Adrenaline μg.	N/A	*Nor*-adrenaline μg.	Adrenaline μg.	N/A
14	0.05	0.015	3.3	0.015	0.01	1.5	0.015	0.005	3.0
15	0.04	0.01	4.0	0.01	0.005	2.0	0.015	0.005	3.0
16	0.04	0.01	4.0	0.005	0.0025	2.0	0.003	0.001	3.0
17	0.03	0.005	6.0	0.005	0.0025	2.0	0.01	0.0025	4.0
18	0.06	0.01	6.0	0.01	0.005	2.0	0.01	0.0025	4.0
19	0.06	0.01	6.0	0.0025	0.0013	2.0	0.02	0.005	4.0
20	0.02	0.005	4.0	0.0075	0.005	1.5	0.003	0.001	3.0
Mean of 7 experiments ..			4.8			1.9			3.4
Mean of 13 experiments ..			5.5			1.7			4.1

on the first day for 29 experiments in Table I. During perfusion with ephedrine the ratio fell to 1.7, and later when the ephedrine perfusion was discontinued the ratio rose again to 4.1. Thus the observations were consistent with the suggestion that the high ratio for N/A observed at first might be due to more rapid destruction of *nor*adrenaline than of adrenaline.

Comparison of Corbasil with adrenaline.—The results, however, did not necessarily indicate that the enzyme involved was amine oxidase. It seemed probable that the addition of ephedrine to the perfusion fluid would be likely to reduce the action of whatever enzyme was present, since the structure of ephedrine would enable it to compete with *nor*adrenaline or adrenaline for any adrenolytic enzyme.

We therefore attempted to carry the investigation further by using Corbasil, which differs from adrenaline simply by the attachment of a methyl group to the α carbon atom. This substance, like ephedrine, can combine with and inhibit the action of amine oxidase, but it is not destroyed by it. We compared Corbasil with adrenaline, just as we had compared *nor*adrenaline with adrenaline, by determining amounts which produced the same vasoconstriction before, during, and after perfusion with ephedrine.

When Corbasil was compared with adrenaline it was found that in the fresh preparation Corbasil was often without vasoconstrictor action except in relatively large amounts. When the perfusion had continued for 1.5 hr., the vessels were then more sensitive. The change is shown in Table III in which the results in 9 experiments where the ratio was determined at once are shown side by side with 7 experiments in which the ratio was determined after 1.5 hr. In both groups the mean amount of adrenaline was almost the same, but in the second group the mean amount of Corbasil was about a quarter of that in the first, showing that the difference in the C/A ratio in the two groups was due to the increase in sensitiveness to Corbasil after 1.5 hr. perfusion.

The effect of adding ephedrine to the perfusion fluid was therefore determined in those experiments in which the ratio between the amounts of Corbasil and adrenaline having equal constrictor effects was observed at the end of 1.5 hr. perfusion. The

TABLE III

AMOUNTS OF CORBASIL AND ADRENALINE PRODUCING THE SAME VASOCONSTRICTION IN RABBIT EAR VESSELS

Experiments in which C/A ratio was determined as soon as perfusion began				Experiments in which C/A ratio was determined after 1.5 hr. perfusion			
Exp.	Corbasil μg.	Adrenaline μg.	C/A	Exp.	Corbasil μg.	Adrenaline μg.	C/A
21	0.6	0.02	30	30	0.025	0.0025	10
22	0.8	0.02	40	31	0.04	0.01	4
23	0.01	0.001	10	32	0.2	0.02	10
24	0.02	0.001	20	33	0.025	0.0025	10
25	0.005	0.00075	6.6	34	0.03	0.005	6
26	0.2	0.005	40	35	0.015	0.002	8
27	0.026	0.002	13	36	0.03	0.004	7.5
28	0.03	0.002	15				
29	0.045	0.005	9				
Mean	0.193	0.0063	20.4		0.052	0.0066	8.0

TABLE IV

AMOUNTS OF CORBASIL AND ADRENALINE PRODUCING THE SAME VASOCONSTRICTION; OBSERVATIONS BEFORE PERFUSION WITH EPHEDRINE WERE MADE ONLY WHEN THE PERFUSION WITH LOCKE'S SOLUTION HAD CONTINUED FOR 1.5 HR.

Exp.	Before perfusion with ephedrine			During perfusion with ephedrine (10 μg./ml.)			After perfusion with ephedrine		
	Corbasil μg.	Adrenaline μg.	C/A	Corbasil μg.	Adrenaline μg.	C/A	Corbasil μg.	Adrenaline μg.	C/A
37	0.04	0.003	13	0.06	0.006	10	0.02	0.002	10
38	0.04	0.003	9	0.05	0.0045	9	0.06	0.0075	8.5
39	0.03	0.009	3.5	0.045	0.015	3	0.03	0.0075	4
40	0.01	0.001	10	0.03	0.01	3	0.013	0.0025	5
41	0.015	0.002	7	0.02	0.002	10	0.02	0.003	6
42	0.05	0.0045	11	0.05	0.004	12	0.02	0.002	10
43	0.0025	0.0005	5	0.018	0.003	6	0.056	0.008	7
Mean			8.3			6.9			6.9
Mean of 14 experiments			8.15			7.6			8.1

results are shown in Table IV, and are difficult to interpret. They differ from those in Table II in that during perfusion with ephedrine the vessels did not become more sensitive to adrenaline. The results in Table II were in full confirmation of the finding of Gaddum and Kwiatkowski that in the presence of ephedrine the vessels become more sensitive to adrenaline, for in each experiment a smaller amount of adrenaline was injected during the ephedrine perfusion. In Table IV, such an increased sensitiveness to adrenaline during perfusion with ephedrine was never observed. The mean ratio of amounts of Corbasil and of adrenaline which caused the same constriction did not alter appreciably; it was 8.3 before ephedrine was added to the perfusing fluid, and 6.9 during perfusion with ephedrine; it remained at 6.9 after

perfusion with ephedrine ceased. This result was clearly unlike the result in Table II, which showed that ephedrine reduced the ratio between the amount of *nor*adrenaline and the amount of adrenaline which produced the same constriction.

The failure of ephedrine to modify the Corbasil-adrenaline ratio supported the explanation of the modification of the *nor*adrenaline ratio. It remained, however, to obtain evidence that amine oxidase destroyed *nor*adrenaline more rapidly than it destroyed adrenaline.

Experiments with amine oxidase

Oxygen uptake.—When adrenaline is incubated with amine oxidase, oxygen is consumed and adrenaline disappears at a rate which is about the same as that when *nor*adrenaline is used instead of adrenaline. Results obtained in this way do not suggest any appreciable difference in the reactions of adrenaline and *nor*adrenaline with amine oxidase.

There is, however, the possibility that if both substrates are present together amine oxidase may then display a greater affinity for one of them, and this may be oxidized more rapidly than the other. If a mixture of equal parts of adrenaline and *nor*adrenaline is incubated, it may then happen that the relative proportion of the two will change, and ultimately only one may still be present. A method for determining the relative proportion of adrenaline and *nor*adrenaline in a mixture has been described by Burn, Hutcheon, and Parker (1950).

In the following experiments a washed suspension of acetone-dried liver was used; such suspensions have high amine-oxidase activity (Blaschko and Hawkins, 1950). The suspensions were incubated in an atmosphere of oxygen at 37° in manometer flasks with adrenaline or *nor*adrenaline, or a mixture of equal parts of both amines, and the oxygen uptake was recorded. Each flask contained 1.4 ml. of the liver suspension, containing 140 mg. of liver powder, and 0.2 ml. of 0.1 M-semicarbazide, in order to prevent further oxidation of the primary products of oxidation. In each flask, the total volume of fluid was brought up to 2.0 ml. by adding 0.4 ml. of a 0.025 M-solution of adrenaline or of *nor*adrenaline, or 0.2 ml. of each of these solutions. The amine concentration at the beginning of the incubation was therefore 0.005 M; the adrenaline-*nor*adrenaline mixtures contained 211 μg. of *nor*adrenaline plus 229 μg. of adrenaline per ml.

The amount of oxygen expected to have been taken up at the end of the oxidation reaction was 112 μl. O_2. Actually, the oxygen uptake did not come to a complete standstill, but continued very slowly, probably owing to non-enzymic oxidation. The course of the O_2 uptake is shown in Fig. 2. In Fig. 2 the initial rate of uptake with *nor*adrenaline is slightly greater than that of adrenaline, but this difference was not observed in all experiments.

Fig. 2 also shows the O_2 uptake in three flasks which contained *nor*adrenaline-adrenaline mixtures. The incubation of these mixtures was not followed to completion. The incubation of the first of these samples was only continued until about a quarter of the theoretical amount of oxygen had been taken up. The enzyme was then rapidly removed by centrifugation; the supernatant was acidified and boiled. The second sample was incubated until about a half, and the third sample until about three-quarters, of the theoretical oxygen uptake was observed; the samples were likewise centrifuged, acidified, and boiled when incubation was terminated.

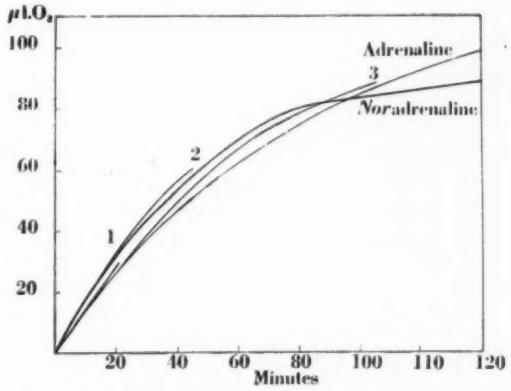

FIG. 2.—This is a record showing oxygen uptake during the incubation with rabbit liver powder of (*a*) adrenaline, (*b*) *nor*-adrenaline, (*c*) mixtures of equal parts of adrenaline and *nor*adrenaline; the incubation of these mixtures was interrupted at the points indicated, (1), (2), and (3). The mixtures were then examined on the blood pressure and nictitating membrane of the spinal cat. We are indebted to Dr. H. Blaschko for this Figure.

More rapid disappearance of nor*adrenaline*.—The proportion of adrenaline and *nor*adrenaline in the incubated samples was then determined by observations in the spinal cat. The method depends on the observation that, while adrenaline causes a contraction of the nictitating membrane, *nor*adrenaline has little or no action upon it. The intravenous injection of a mixture of *nor*adrenaline and adrenaline causes a rise of blood pressure and a contraction of the nictitating membrane, and, from the ratio of the contraction of the membrane to the rise of blood pressure, the proportion of the two amines can be determined by matching with known mixtures. In each experiment on a spinal cat the effect of the incubated samples was compared with that of known mixtures of the two solutions from which the mixtures for incubation were prepared.

The results showed that in the course of incubation, while the total pressor activity fell in proportion to the oxygen consumed, the percentage of adrenaline in the mixture rose and the percentage of *nor*adrenaline declined. The observations made are given in Table V.

TABLE V

INCUBATION OF A MIXTURE CONTAINING EQUAL PARTS OF ADRENALINE AND *nor*ADRENALINE WITH AN ACETONE-DRIED POWDER OF LIVER

Exp.	Liver from	Percentage of adrenaline in mixture at different stages of incubation; incubation arrested when O_2 consumption was		
		¼ complete	½ complete	¾ complete
44	Rabbit	58	88	100
45	,,	48	77	79
46	,,	—	71	90
47	,,	52	72	88
48	,,	59	72	88
49	Cat	64	70	76

In experiment 44, before incubation began, each mixture contained, as already stated, about 440 μg. active base per ml. The oxygen consumption was a quarter complete after 30 min. in one mixture; incubation was arrested and the mixture then had a pressor action equivalent to 320 μg. adrenaline per ml., and of this pressor action 58 per cent was due to adrenaline. In a second mixture the oxygen consumption was half complete after 60 min.; the mixture then had a pressor action equivalent to 200 μg. adrenaline per ml., and of this pressor action 88 per cent was due to adrenaline.

Finally, in a third mixture, oxygen consumption was about three-quarters complete after 155 min.; the mixture then had a pressor action equivalent to 61 μg. adrenaline per ml., and of this pressor action 100 per cent was due to adrenaline.

Exps. 44 to 48 were carried out with an acetone powder from rabbit liver, Exp. 49 with a similar powder from cat liver. Thus the results were consistent in showing that under the conditions of the experiment *nor*adrenaline was destroyed more rapidly than adrenaline, presumably because of a greater affinity of the enzyme for the former substance.

Discussion

The experiments described throw some light in the first place on the varying figures which have been obtained by several workers for the relative vasoconstrictor action of *nor*adrenaline and adrenaline in the vessels of the rabbit ear. When the ear is freshly perfused *nor*adrenaline has relatively little constrictor action compared with adrenaline; when the perfusion has continued overnight its constrictor action is much closer to that of adrenaline, though still less.

Since it has been found by Schapira (1945) that amine oxidase is present in the rabbit ear and by Thompson (unpublished) that it is present in the ear vessels, it seemed possible that the increased constrictor action of *nor*adrenaline after 24 hr. was due to diminished destruction by amine oxidase. Now ephedrine is known to inhibit the action of amine oxidase (Blaschko, Richter, and Schlossmann, 1937), and we therefore compared the ratio of amounts of *nor*adrenaline and adrenaline which produced the same constriction, before, during, and after perfusion with ephedrine. We observed that ephedrine caused a drop in the ratio, and that the ratio rose again when the ephedrine was discontinued. This result is consistent with the explanation that inhibition of amine oxidase by ephedrine renders the vessels more sensitive to adrenaline, but still more sensitive to *nor*adrenaline.

Now Corbasil is a substance similar to adrenaline with a methyl group attached to the α carbon atom of the side chain. It is therefore not destroyed by amine oxidase, but it inhibits the action of this enzyme. We determined the effect of ephedrine perfusion on the ratio of the amounts of Corbasil and adrenaline which produced equal constriction. We found that ephedrine had no effect on this ratio, and indeed in these experiments ephedrine appeared to have no effect at all since it failed to sensitize the vessels to adrenaline. The failure of ephedrine to exert an action could be explained by supposing that the enzyme was already inhibited by the successive injections of Corbasil which were previously given. However this may be, the observations showed that, while ephedrine lowered the ratio of amounts of *nor*adrenaline and adrenaline which produced equal constriction, it did not modify the corresponding ratio of amounts of Corbasil and adrenaline.

The fall in the ratio of amounts of *nor*adrenaline and adrenaline which caused equal vasoconstriction, produced by including ephedrine in the perfusion fluid, suggested that amine oxidase destroyed *nor*adrenaline more readily than it destroyed adrenaline. Evidence for this was lacking. When preparations containing amine oxidase act on adrenaline or on *nor*adrenaline, the rate at which oxidation proceeds is similar for the two substances, as was observed by Blaschko, Richter, and Schlossmann (1937). We have confirmed this finding. If, however, the two substances are placed together in the same manometer flask in the presence of an acetone-dried

preparation of rabbit liver, or of cat liver, the *nor*adrenaline disappears at a faster rate than the adrenaline. Mixtures of equal amounts in the course of incubation were found to decline in percentage of *nor*adrenaline present and to increase in percentage of adrenaline.

All these observations combine in favour of the supposition that, in the rabbit ear vessels, adrenaline and *nor*adrenaline are destroyed by amine oxidase, which is known to be present there, and that the relative weakness of *nor*adrenaline in the freshly perfused ear is due to its more rapid destruction by this enzyme. The observations, however, cannot be said to prove this point since an increased sensitivity of the vessels need not be due to the action of amine oxidase. For example, the increased sensitivity to Corbasil during the first hour's perfusion (Table III), and the increased sensitivity to adrenaline after cessation of perfusion with ephedrine (Table II), cannot be explained by the action of amine oxidase.

Summary

1. Amine oxidase, in the form of an acetone-dried preparation of rabbit or cat liver, has a greater affinity for *nor*adrenaline than for adrenaline, since, when it is allowed to act upon a mixture of equal parts of adrenaline and *nor*adrenaline, the percentage of adrenaline rises during the fall of the total quantity of pressor amines.

2. When a rabbit ear is perfused with Locke's solution, and when constrictor effects are obtained by injecting small quantities of *nor*adrenaline and of adrenaline into the perfusing fluid, the ratio of amounts of the two substances which produce the same constriction is high, indicating that *nor*adrenaline is relatively weak. When the perfusion has continued for 24 hr., *nor*adrenaline more nearly approaches adrenaline in constrictor action.

3. Since amine oxidase is present in rabbit ear vessels, these observations could be explained by supposing that at first amine oxidase is very active, and its greater affinity for *nor*adrenaline is responsible for the weak constrictor action of this substance, but that after 24 hr. amine oxidase becomes less active.

4. Support is given to this explanation by the fact that the addition of ephedrine, which inhibits amine oxidase, to the perfusing fluid increases the constrictor action of *nor*adrenaline more than it increases that of adrenaline.

5. Ephedrine, when added to the perfusing fluid, does not modify the relative constrictor action of Corbasil (which is not destroyed by amine oxidase) and adrenaline.

It is a pleasure to thank Dr. H. Blaschko for arranging the experiments in which the pressor amines were incubated with amine oxidase, and for Fig. 2.

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REVERSAL OF THE VASCULAR RESPONSE TO ACETYLCHOLINE AND ADRENALINE

BY

J. H. BURN AND JUDITH ROBINSON

From the Department of Pharmacology, Oxford University

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The phenomenon of the reversal of the vasomotor action of adrenaline by extracts of ergot was first described by Dale (1906). It has been studied by many workers since it was originally described, and it has been produced by many substances other than those found in ergot. Little light, however, has been shed on the process involved, partly because most observations have been made by recording the blood-pressure in the anaesthetized or spinal animal. So many different facts modify the blood pressure response that it is difficult to be certain what parts of the cardiovascular system are responsible for the reversal of the effect.

METHOD

We have therefore carried out observations on the vessels of the rabbit ear perfused at a constant head of pressure with Locke's solution at room temperature using the outflow recorder devised by Stephenson (1948) in this laboratory. This apparatus is simple to make from glass tubing, and gives an extremely sensitive record of changes in the size of the vascular channels in the ear.

A fine glass cannula was tied into the central artery at the base of the ear and fluid passed to this cannula by way of the small rubber-capped chamber described by Gaddum and Kwiatkowski (1938). Injections were made into this chamber through the cap, the fluid injected being Locke's solution containing the active agent. The final dilution of the agent in Locke's solution was made just before injection. When the effect observed was dilatation, we always gave control injections to ensure that this was not due to the volume of the injected fluid. When the ear vessels are perfused with a solution containing histamine, for example, they become very sensitive to the mechanical effect of injecting fluid.

Responses to acetylcholine

Spontaneous change during perfusion.—When acetylcholine (ACh) is injected into the cannula during the first few hours of perfusion the vessels nearly always respond by dilatation. The amounts sufficient to produce the effect vary from 1 to 20 μg., though dilatation may be caused by 100 μg. As perfusion continues, the dilatation in response to a given dose declines and may disappear; when a larger dose is injected constriction is then seen, and from this point onwards all injections of acetylcholine cause constriction. An example of the change is given in Fig. 1. The time which elapses between the beginning of the perfusion and the appearance of the constrictor response varies in different ears. In our experience the dilator response usually persists during the first 6 hr.; the constrictor response is seen when injections are made the

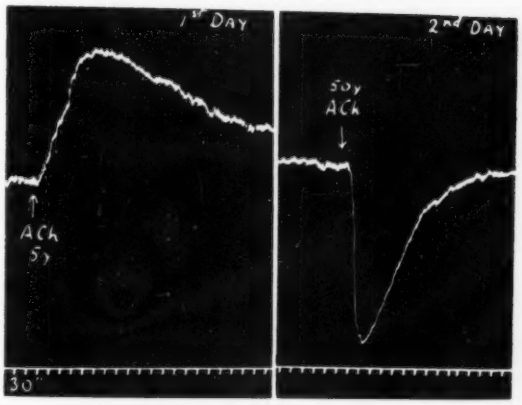

FIG. 1.—Record of outflow from rabbit ear vessels, showing increase caused by the injection of 5 μg. acetylcholine (ACh) during perfusion on 1st day, and on the right the decrease caused by the injection of 50 μg. acetylcholine on 2nd day.

next day, the perfusion having continued overnight. Sometimes, however, the dilator response persists during the second day, and then gives place to a constrictor response on the third day. Sometimes the dilator response disappears during the first day, and occasionally constriction is the response which is seen at the beginning of the perfusion. The change in the response is always in the direction of dilatation to constriction. We have never observed a spontaneous change in the reverse direction. In nineteen consecutive experiments we observed a dilator response on the first day in sixteen. The change in a succession of eleven experiments is recorded in Table I. During the first day the response to acetylcholine was dilatation in ten experiments; in the remaining experiment acetylcholine caused constriction. The perfusion was continued on the second day, and acetylcholine then caused constriction in nine experiments; in the remaining two experiments acetylcholine caused dilatation, as on the first day. In these two experiments acetylcholine caused constriction on the third day. All third-day responses were constriction.

Effect of dose.—When the response to acetylcholine is dilator, it is usually obtained with small doses; when, on the other hand, the response is constrictor it is usually obtained with larger doses. This difference, which is illustrated in Fig. 1, suggests

TABLE I

EFFECT OF ACETYLCHOLINE ON EAR VESSELS AT DIFFERENT STAGES OF PERFUSION

Exp.	Response and dose: D = dilatation, C = constriction		
	1st day	2nd day	3rd day
1	D 100 μg.	C 100 μg.	
2	D 10	C 20	
3	C 50	C 200	
4	D 20	C 20	
5	D 20	C 20	C 25 μg.
6	D 10	C 10	
7	D 1	D 2.5	C 50
8	D 5	C 10	
9	D 2.5	C 50	
10	D 4	D 4	C 10
11	D 10	C 20	

that the response is determined by the size of the dose. In some experiments dilatation has been produced during the first day's perfusion by a large dose, 0.1 mg., and constriction has been produced by the same dose during the second day's perfusion. In Exps. 1, 4, 5, and 6 of Table I constriction was produced on the second day by the same dose which produced dilatation on the first day. This indicates that the response does not depend on the dose. In other experiments, however, in which a dilator response was recorded to a certain dose, a greater dose caused a transitory constriction preceding the dilatation.

Restoration of a dilator response.—When the response to acetylcholine has become constrictor, the addition of adrenaline or of *nor*adrenaline to the Locke's solution perfusing the ear restores the dilator action of acetylcholine. This effect is illustrated in Fig. 2, which records responses obtained on the second day of perfusion. In the upper part of Fig. 2, 10 μg., 50 μg., and 100 μg. acetylcholine were injected in succession, with increasing constrictor action. *Nor*adrenaline was then added to the perfusing fluid in a concentration of 10^{-7}. The response to the injection of acetylcholine then became dilator, as shown in the lower part of Fig. 2. The dilatation was produced by the injection of 1 μg. The injection of 10 μg. produced a much larger dilatation.

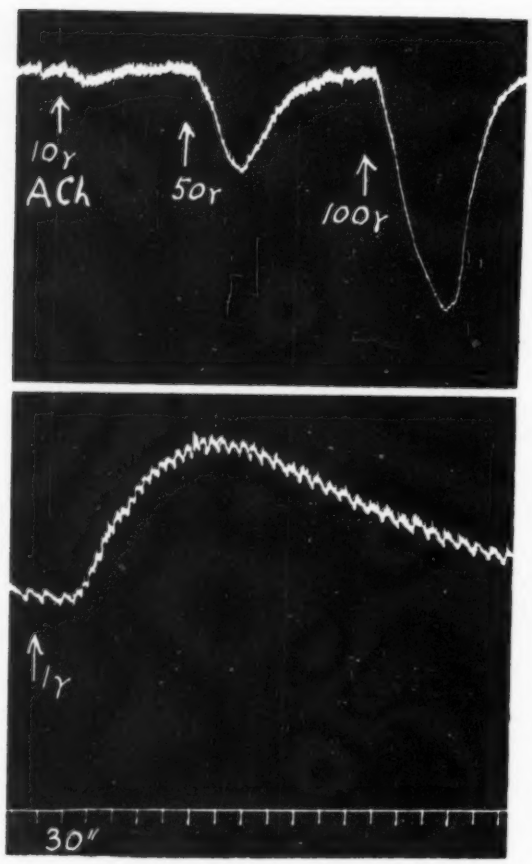

FIG. 2.—Records during perfusion on 2nd day. Decrease in outflow caused by 10 μg., 50 μg., and 100 μg. acetylcholine. *Nor*adrenaline was then added to the Locke's solution, the concentration being 10^{-7}. During perfusion with this solution, dilatation was produced by injecting 1 μg. acetylcholine.

Restoration by histamine.—Experiments were also carried out to determine the effect of histamine, since it is known from the observations of Schmiterlöw (1948) that histamine is present in the arterial walls of oxen. Fig. 3 shows observations made on the second day of perfusion; in (*a*) the injection of 0.15 mg. acetylcholine produced vasoconstriction; histamine acid phosphate was then added to the perfusion fluid in a concentration of 10^{-5} (histamine base). This addition had a slight effect in constricting the vessels, but very little. The response to injections of 0.15 mg. acetylcholine was steadily modified, however. At first, as shown in Fig. 3 (*b*) and (*c*), the constrictor action of acetylcholine was progressively augmented. As the perfusion continued, the constriction became shorter in duration and was

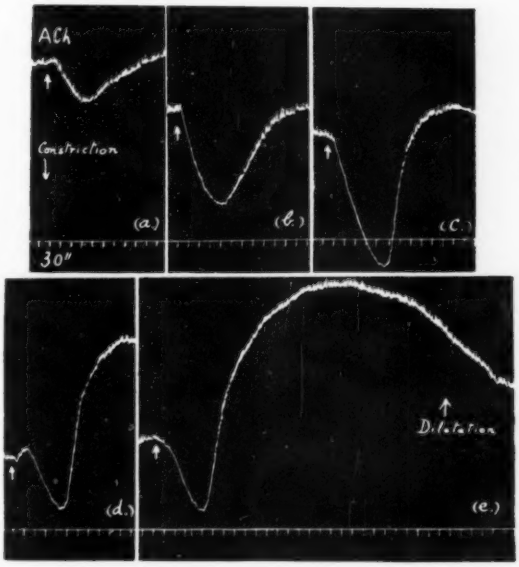

FIG. 3.—Records during perfusion on 2nd day. In (*a*) the decrease in outflow caused by injecting 150 μg. acetylcholine is recorded. Histamine acid phosphate was then added to the Locke's solution, the concentration being 10^{-5} (histamine base). In (*b*) 150 μg. acetylcholine caused a greater constriction, and in (*c*) it caused a still greater one. In (*d*) the injection caused constriction followed by dilatation, and in (*e*) the main effect of the same injection was dilatation.

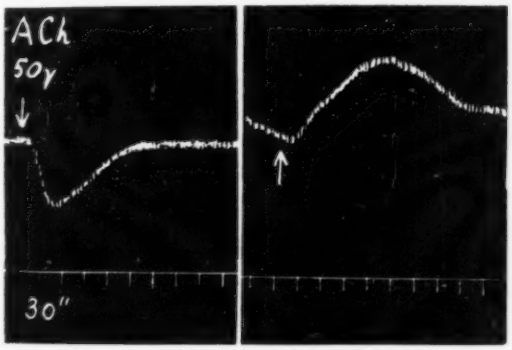

FIG. 4.—Records on 2nd day. Constrictor effect of 50 μg. acetylcholine. During perfusion with Locke's solution containing histamine 10^{-5}, the injection of 50 μg. acetylcholine caused dilatation.

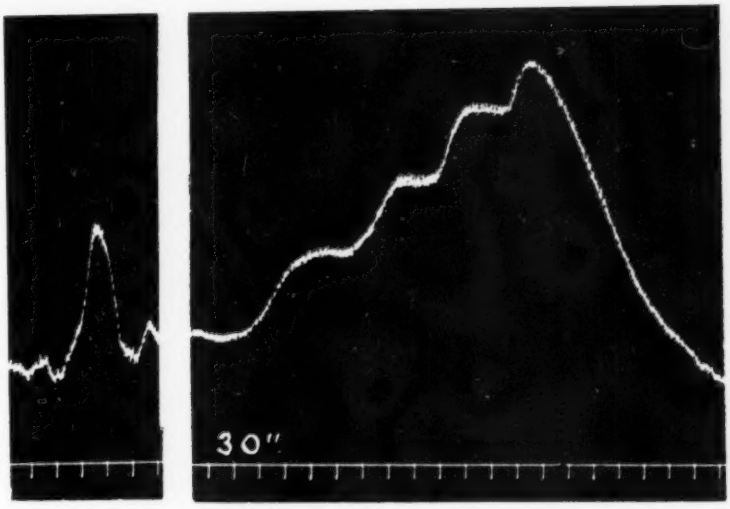

FIG. 5.—Record showing two examples of spontaneous changes in outflow during perfusion on the 1st day, when the perfusing fluid contained 10^{-5} histamine.

followed by dilatation. In Fig. 3 (*e*), the principal effect of the injection of 0.15 mg. acetylcholine was dilatation. When 0.05 mg. acetylcholine was next injected, dilatation only was recorded.

The same sequence of changes was observed in several experiments. In some only the first phase of potentiation of the constrictor action of acetylcholine was obtained. In others simple reversal was recorded as in Fig. 4.

In all these experiments the effect of adding histamine to the Locke's solution was determined during perfusion on the second day. When histamine was added to the perfusion fluid on the first day, it led to the occurrence of spontaneous changes in the vessels (see Fig. 5), and a great sensitivity to the mechanical effect of injections of Locke's solution, the injection of 0.05–0.1 ml. causing a large dilator response.

Response to adrenaline

Initial dilator response.—In a previous paper Burn and Dutta (1948) described the unusual results they obtained in one experiment on the perfused rabbit's ear, in which they repeatedly observed dilator responses to adrenaline and histamine as well as to acetylcholine during the first day's perfusion. On the second day the responses to all three substances were constrictor. We have now observed several more examples of an initial dilator response to adrenaline at the beginning of a perfusion. This dilator response has not persisted throughout the first day's perfusion, but has rapidly diminished and then given place to the usual constrictor response. Examples of this change are given in Figs. 6 and 7. In Fig. 6 when 0.01 μg. adrenaline was injected it produced first of all the dilatation shown at the top of the figure. A control injection of fluid was given; it had no effect. Later injections of 0.01 μg. adrenaline caused

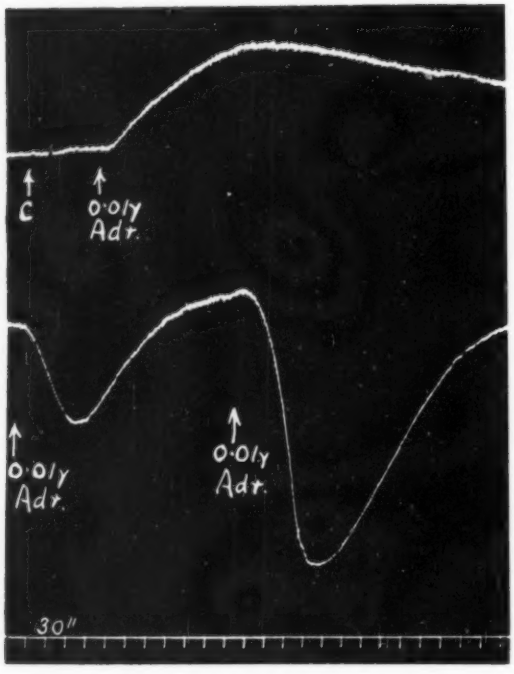

FIG. 6.—Record at beginning of perfusion showing the dilator effect of 0.01 μg. adrenaline injected in 0.1 ml. At C, 0.1 ml. perfusion fluid was injected. The lower tracings show the effects of injecting 0.01 μg. adrenaline a little later. The dilator response changed spontaneously to a constrictor response.

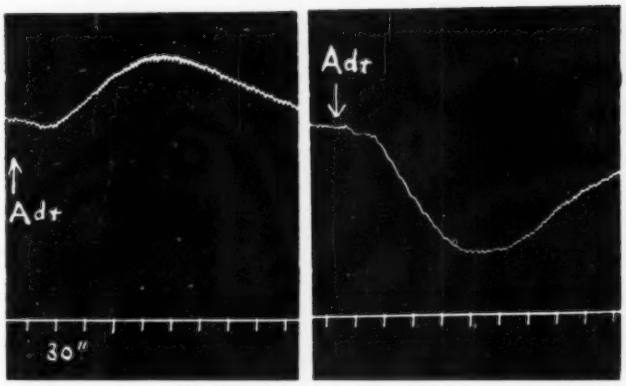

FIG. 7.—Record shows a similar change to that in Fig. 6. 0.005 μg. adrenaline produced dilatation at first, but after 30 min. it caused constriction.

in succession the small and then the larger constriction seen in the lower part of the figure. In Fig. 7 an injection of 0.005 μg. adrenaline caused dilatation, whereas the same dose injected 32 min. later caused the usual constrictor response. In this experiment the same change in response was observed when *nor*adrenaline was injected in a dose of 0.01 μg. Thus, when a long series of experiments is carried out in which rabbit ears are perfused, it is found that in a small proportion of these the initial effect of adrenaline, like the effect of acetylcholine, is dilator.

Reversal by ephedrine.—It was shown by Gaddum and Kwiatkowski (1938) that, when ephedrine is added to the fluid perfusing the vessels of the rabbit ear, the constrictor action of both adrenaline and of sympathetic stimulation is potentiated. We have recently carried out many experiments (Burn and Robinson, 1951) in which we have determined the effect of adding ephedrine to the perfusing Locke's solution, and in one of these we obtained an unusual result. The vessels of the ear in this experiment were found to be very constricted when the perfusion began, and, even when the Marriotte bottle containing Locke's solution was raised about 1.3 metres

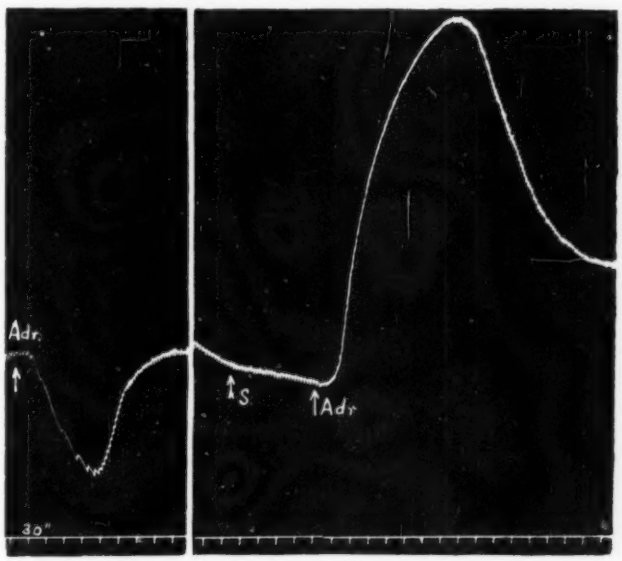

FIG. 8.—Ear perfusion on 1st day. On the left is shown the constrictor action of 0.003 μg. adrenaline. Ephedrine hydrochloride was then added to the Locke's solution in a concentration of 10^{-5}; during perfusion with this solution 0.1 ml. perfusing fluid was injected at S, and then 0.002 μg. adrenaline. This last injection caused vasodilatation.

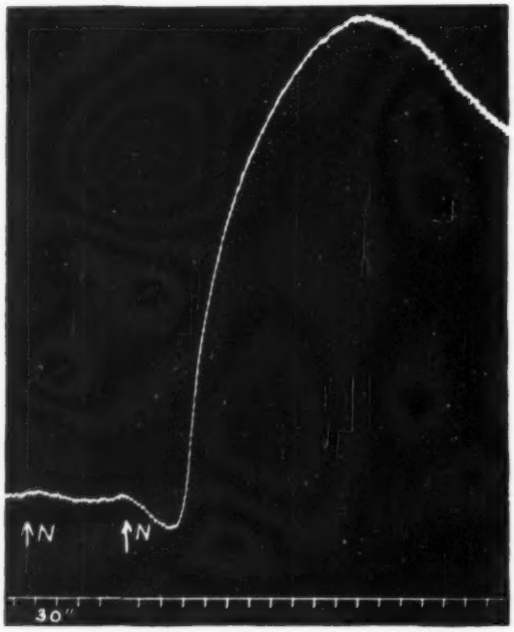

FIG. 9.—In the same experiment as shown in Fig. 8, during perfusion with Locke containing ephedrine, 0.002 μg. *nor*adrenaline and then 0.008 μg. *nor*adrenaline were injected. The last injection caused vasodilatation.

above the ear, little fluid passed through the vessels. The preparation was left for some time under this high pressure, and after 1 hr. fluid began to flow out of the veins. The pressure was then reduced. When injections of adrenaline were made, the usual constriction was produced as shown in Fig. 8. Ephedrine hydrochloride was then added to the Locke's solution in a concentration 10^{-5}, and the perfusion was continued with this solution. The injection of the same amount of adrenaline as before now produced dilatation as shown in Fig. 8. Thus in the presence of ephedrine the constrictor action of adrenaline was reversed to dilatation. A similar reversal took place of the action of Corbasil, a substance resembling adrenaline with the addition of a $-CH_3$ group to the α carbon atom. When perfusion began without the addition of ephedrine to the Locke's solution, 0.025 μg. Corbasil caused vasoconstriction similar in magnitude to that caused by 0.003 μg. adrenaline; after the addition of ephedrine, the same dose of Corbasil produced vasodilatation like that caused by 0.002 μg. adrenaline. *Nor*adrenaline was not injected before the addition of ephedrine to the perfusion fluid; during perfusion with ephedrine it caused vasodilatation as shown in Fig. 9.

DISCUSSION

The experiments serve in the first place to emphasize what is not well known, that acetylcholine has two actions on blood vessels and can cause vasoconstriction as well as vasodilatation. The existence of this double effect caused controversy between Dale (1914) and Reid Hunt (1915), for, whereas Dale observed that acetylcholine dilated the vessels of the rabbit ear in a dose of 5 μg., Reid Hunt observed either no effect or vasoconstriction in a dose of 0.2 mg. Our experiments show that when rabbit ear vessels are perfused with Locke's solution the early response to acetylcholine is almost always vasodilatation, but that when the perfusion has continued for a period measured in hours the response becomes vasoconstriction. The response can be converted once more to vasodilatation by adding to the perfusing fluid either adrenaline or *nor*adrenaline in concentrations of 10^{-7}, or histamine in a concentration of 10^{-5}. The experiments in which the vasodilator response was restored by histamine were undertaken because it has been shown by Schmiterlöw (1948) that the blood vessels of the ox contain not only *nor*adrenaline but also acetylcholine and histamine. It is possible that the same three substances are present in the vessels of the rabbit ear;

if this is so it is also possible that when the ear is continuously perfused the amount of any or all of these substances declines, and that the change in the reaction of the vessels to acetylcholine is due to this loss.

However this may be, when the perfusion of an ear has continued long enough for the reaction to acetylcholine to become constrictor, the addition of histamine to the perfusion fluid changes the reaction back to dilatation. Histamine in a concentration of 10^{-5} has little constrictor action of its own, and the reversal of the action of acetylcholine is not due to increased tone, because the initial effect of histamine is to augment the constrictor action of acetylcholine. This finding was confirmed many times, and in some experiments was the only effect which the addition of histamine to the perfusion fluid was observed to produce.

In these experiments histamine was added to the perfusing Locke's solution on the second day of the perfusion. When histamine was added on the first day it had a peculiar effect in producing spontaneous changes in the vessels. The outflow from the veins increased and declined rhythmically, and if a small injection of the perfusing fluid was made the vessels responded by a transient dilatation to such an extent that it became impossible to study the effect of other substances. This action of histamine seems to be of some interest.

The observation was previously made by Burn and Dutta (1948) that various substances, among others quinidine, pethidine, and benadryl, when injected into the arterial cannula during a perfusion of the vessels of the rabbit ear with Locke's solution caused vasoconstriction. When a known reversing agent, benzylimidazoline, was added to the perfusing fluid, in a concentration which reversed the constrictor action of adrenaline, the constrictor action of these different substances was also reversed. Thus it was shown that quinidine, pethidine, and benadryl possess a vasoconstrictor action which must closely resemble that of adrenaline, since when the action of adrenaline is reversed their action is reversed also.

Burn and Dutta (1948) found in addition that quinidine, pethidine, and benadryl were able to act as reversing agents, for when any one of them was added to the fluid perfusing the rabbit's ear in a concentration of 10^{-5}, the constriction caused by the injection of adrenaline into the arterial cannula was replaced by dilatation. Their experiments demonstrated that adrenaline-like substances were themselves reversing agents, and they therefore suggested that adrenaline reversal was essentially due to competition by the reversing agent for the receptor to which adrenaline attaches itself. The evidence now obtained gives considerable support to this view, for an experiment is described in which ephedrine added to the perfusing fluid was found to reverse the action of adrenaline, of Corbasil, and also of *nor*adrenaline. Thus a substance generally accepted as exerting many of its effects by competition with adrenaline has been shown to reverse the action of adrenaline from constriction to dilatation.

The question then arises how a competing substance can produce reversal. Current theory, which has recently been discussed (Burn, 1950), assumes that there are two kinds of receptors, the one responsible for constriction and the other for dilatation. There is, however, no evidence of two kinds of receptors for acetylcholine, which has both effects in the rabbit ear vessels. In other tissues also acetylcholine has been shown to have two effects; thus Bülbring and Burn (1949) have shown that when the isolated auricles of the rabbit heart cease to contract after beating for 25–30 hr. the normal

inhibitory action of ACh is reversed to a stimulant action; when a certain degree of activity has been re-established, the normal inhibitory action reappears. Similarly, Burn and Vane (1949) have shown that in the isolated intestine of the rabbit or the isolated uterus of the rat the stimulant action of smaller concentrations is replaced by an inhibitory action of higher concentrations. In these tissues the difference in the effect of low and high concentrations of acetylcholine can be more easily explained by calling to mind the effect of acetylcholine concentration on the rate of its hydrolysis by " true " or " specific " cholinesterase. As the concentration of acetylcholine is increased, at first the rate of hydrolysis rises; but after reaching a peak the rate of hydrolysis falls gradually to zero. In order that hydrolysis may take place each molecule of acetylcholine is supposed to be attached to the molecule of enzyme at two points A and B. When excess of acetylcholine is present, two molecules of it may attach themselves, one by point A and the other by point B. Hydrolysis cannot then occur, and the enzyme is inhibited.

In a simple vascular preparation, like the system in the rabbit ear, dilatation may be an inhibition of this kind. When the relative number of free receptors and of molecules of acetylcholine is such that there is an excess of acetylcholine, attachment of the molecules may take place in such a way that the acetylcholine cannot be hydrolysed, and then the receptors will be blocked. Dilatation will follow. On the other hand, when there is no excess of acetylcholine, the attachment of the molecules to the receptors will cause stimulation attended by splitting of the ester.

The reversal of the constrictor action of adrenaline by ephedrine is capable of interpretation on these lines. Usually ephedrine does not reverse the constrictor action, but rather increases it. The potentiation was first explained by Gaddum and Kwiatkowski (1938) as due to inhibition of amine oxidase, and support for this view has recently been obtained (Burn and Robinson, 1951). Thus the potentiation occurs because a greater number of adrenaline molecules are free to combine with the receptors.

We can imagine that in some experiments perfusion of the vessels with ephedrine would have another effect. If the number of enzyme molecules was small, ephedrine might combine with all of them, and then combine with some of the receptors in addition. The effect of this would be to reduce the number of free receptors and so to reduce the sensitivity of the vessels to adrenaline. In the course of 55 perfusions with ephedrine we observed that the amount of adrenaline required to produce a given constriction was increased in 16 perfusions, and in some of these the decrease in sensitivity was sixfold.

In the experiment in which ephedrine caused reversal we may suppose that the perfusion with ephedrine led to combination of ephedrine with all enzyme molecules and also with so many of the receptors that an amount of adrenaline which previously produced constriction was in excess for these receptors. Therefore the molecules of adrenaline became attached to them in such a way that they could not exert a stimulant action, and served only to block the receptors, with resulting inhibition or dilatation.

Experiments in which the first few injections of adrenaline into the perfused ear vessels cause vasodilatation are rare, and are easily missed unless a larger series of experiments is performed. We suppose that in these exceptional experiments very few receptors are free, and that small amounts of adrenaline cause inhibition of the vascular tone. After perfusion has continued for a time, which may be short, the

usual constrictor action of adrenaline is seen, probably because the substances which blocked the receptors are washed away.

These changes in the response to adrenaline resemble the changes in the response to acetylcholine. We see that both substances begin by causing dilatation and later cause vasoconstriction; however, the dilator response to acetylcholine is much more persistent than that to adrenaline, while the constrictor response to adrenaline is much more powerful than that to acetylcholine. With both substances the reversal from dilatation to constriction is spontaneous, not requiring the intervention of a third substance, and therefore we think it reasonable to suppose that the reversal takes place because of an increase in the number of free receptors as perfusion goes on, so that there is a rise in the ratio of free receptors to molecules of adrenaline or acetylcholine. This conception fits with the observation that, when a competing substance such as ephedrine is introduced which diminishes the number of free receptors, then constriction is reversed once more to dilatation.

Summary

1. When the vessels of the rabbit ear are perfused with Locke's solution, the injection of acetylcholine at first causes dilatation, but after some hours the injection causes constriction. Larger amounts are usually needed to cause constriction than were needed to cause dilatation.

2. The constrictor response can be reversed once more to dilatation by adding to the Locke's solution either adrenaline or *nor*adrenaline in a concentration of 10^{-7} or histamine in a concentration of 10^{-5}.

3. The first effect of adding histamine to the Locke's solution is to potentiate the constrictor action of acetylcholine; the later effect is to restore the vasodilator response to acetylcholine.

4. When a large series of ears is perfused, occasional preparations respond to adrenaline initially by vasodilatation. After perfusion has continued for an hour or more, the response changes spontaneously to vasoconstriction.

5. In one experiment the addition of ephedrine to the Locke's solution reversed the constrictor action of adrenaline, of Corbasil, and of *nor*adrenaline.

6. The evidence is taken to indicate that when a substance reverses the action of adrenaline from constriction to dilatation it does so by competing with adrenaline for the receptors with which it combines. When most of the receptors are occupied by the competing substance, the molecules of adrenaline, being now in excess in relation to the number of remaining receptors, combine with these receptors in an abnormal manner and block them. Inhibition or dilatation then follows.

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BIOLOGICAL ESTIMATION OF ADRENALINE AND *NOR*ADRENALINE IN TISSUE EXTRACTS CONTAMINATED WITH HISTAMINE OR ACETYLCHOLINE

BY

CARL G. SCHMITERLÖW*

From the Department of Pharmacology, Oxford University

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Various biological methods have been devised for the estimation of adrenaline and *nor*adrenaline in tissue extracts. As these two substances frequently occur together in different proportions it has become necessary to adopt methods which allow the estimation not only of the total amount of pressor substances but also the percentage of adrenaline and *nor*adrenaline in a mixture. Two different ways have been followed. In the first, both these estimations are performed simultaneously and in the same animal, the cat's blood pressure being used as a test of the amount and the nictitating membrane or non-pregnant uterus *in situ* as an indicator of the percentage. The second way involves the use of two (or more) separate preparations, viz., cat's blood pressure and different isolated organs, such as rat's uterus, hen's rectal caecum, cat's intestine, etc. From the results thus obtained the percentage of adrenaline and *nor*adrenaline can be calculated, the principle being that the activity ratio adrenaline/*nor*adrenaline on the cat's blood pressure is correlated with the same ratio obtained from some test object on which the two substances differ significantly from each other in effect (see, e.g., West, 1947; Euler, 1949; Gaddum, Peart, and Vogt, 1949; Burn, Hutcheon, and Parker, 1950).

Both these ways involve the use of the cat's blood pressure for the estimation of the total pressor activity in tissue extracts. Various extraction methods have been adopted, and it should be remembered that figures given for the content of adrenaline and *nor*adrenaline in *tissues* are in fact only figures for the amounts estimated in *extracts* of these tissues. Many of these extracts contain histamine in addition to the pressor substances and this contamination may seriously interfere with the correct determination of the nature of these substances, as was pointed out by Schmiterlöw (1948a, b). Even if the amount of histamine is too small to exert any blood pressure lowering effect it may still cause a liberation of adrenaline from the adrenals of the test animal, and the effect of this released adrenaline is mixed with the effect of the extract itself.

This error can be avoided by tying off the adrenals of the test animal. But the admixture of blood pressure lowering substances may still lead to an erroneous

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impression as regards both total amount and percentage of adrenaline and *nor*adrenaline, as will be shown here.

EXPERIMENTAL

Chloralosed cats.—The blood pressure and the contractions of the normal nictitating membrane were recorded in cats under chloralose (0.07 g. per kg. body weight). The animals were given cocaine in a dose of 3 mg./kg. intramuscularly at the beginning of an experiment in order to render them more sensitive to adrenaline and *nor*adrenaline.

Different mixtures of *nor*adrenaline and histamine were given in order to find the proportion in which the depressor effect of the histamine was completely masked by the pressor effect of the *nor*adrenaline. This mixture, giving a pure rise in blood pressure, caused a fairly strong contraction of the nictitating membrane. When these effects were compared with the action of the same amount of *nor*adrenaline given alone, two facts were observed. Firstly, the rise of blood pressure caused by the mixture was less than that caused by the *nor*adrenaline alone, although the difference was fairly small. Secondly, the mixture caused a much stronger contraction of the nictitating membrane. This is illustrated in Fig. 1.

The same experiment was carried out with mixtures containing *nor*adrenaline and acetylcholine. Similar results were obtained, the difference in response on the nictitating membrane between the mixture and the pure *nor*adrenaline being, however, less pronounced.

These experiments show that, if histamine or acetylcholine is present together with *nor*adrenaline, the latter substance cannot be accurately estimated by this method.

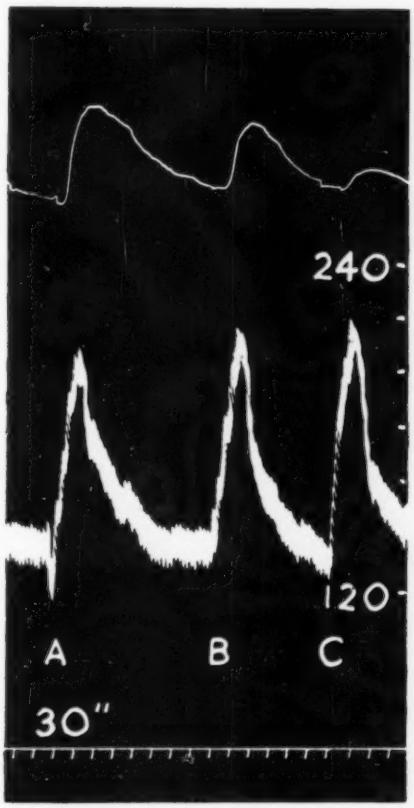

FIG. 1.—Chloralosed cat; cocaine. Normal nictitating membrane above, blood pressure below. A = 2 μg. *l-nor*adrenaline + 1 μg. histamine (base). B = 2 μg. *l-nor*adrenaline + 0.5 μg. histamine. C = 2 μg. *l-nor*adrenaline.

The following experiment was now performed:

1. Testing of the mixtures and pure *nor*adrenaline as described above.
2. Testing of the solutions after removal of the adrenals.
3. Testing after an antihistamine, and finally
4. Testing after atropine.

The results are illustrated in the figures. Fig. 2 shows the effects on a cat (under chloralose and after cocaine) before removal of the adrenals. The mixtures cause a less pronounced rise of blood pressure than *nor*adrenaline alone but a stronger contraction of the nictitating membrane than an equipressor dose of *nor*adrenaline (Fig. 2, D). Note that *nor*adrenaline plus histamine cause a slightly stronger contraction of the membrane than *nor*adrenaline plus acetylcholine. Histamine is obviously more active than acetylcholine in releasing adrenaline from the adrenals.

Fig. 3 (from the same cat as in Fig. 2) shows the effects after the adrenals had been removed. The mixtures now gave contractions of the nictitating membrane equal to that produced by the same amount of *nor*adrenaline alone, but they still gave a smaller rise in blood pressure.

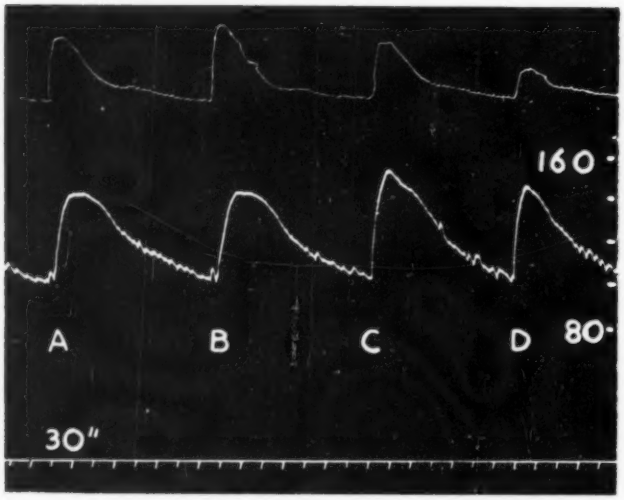

FIG. 2.—Chloralosed cat; cocaine. Normal nictitating membrane above, blood pressure below. A = 5.5 μg. *l-nor*adrenaline + 1 μg. acetylcholine. B = 5.5 μg. *l-nor*adrenaline + 1 μg. histamine. C = 5.5 μg. *l-nor*adrenaline. D = 3 μg. *l-nor*adrenaline.

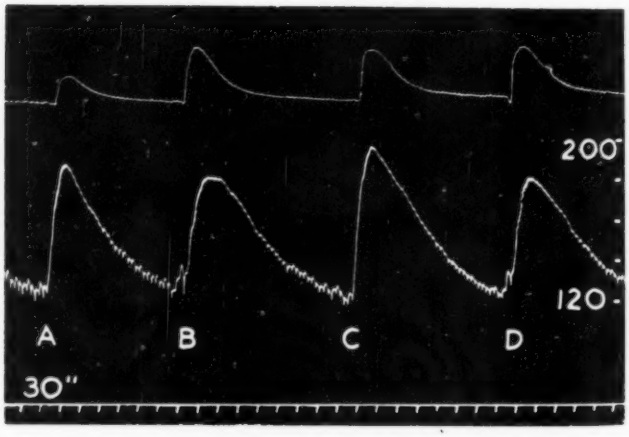

FIG. 3.—Same cat as in Fig. 2. Adrenals now removed. A = 3 μg. *l-nor*adrenaline. B = 5.5 μg. *l-nor*adrenaline + 1 μg. histamine. C = 5.5 μg. *l-nor*adrenaline. D = 5.5 μg. *l-nor*adrenaline + 1 μg. acetylcholine.

If an antihistamine (2 mg./kg. of Lergigan, described by Halpern and Schmiterlöw, 1951) was given the picture was altered. *Nor*adrenaline plus histamine now exerted the same action on both blood pressure and nictitating membrane as the same dose of

*nor*adrenaline alone, whereas *nor*adrenaline plus acetylcholine gave a smaller pressor effect. Finally atropine (0.5 mg. per kg.) was given, and Fig. 4 shows that the mixtures now behaved exactly as *nor*adrenaline alone, i.e., the *nor*adrenaline of the mixture could now be correctly estimated in spite of the admixture of histamine or acetylcholine.

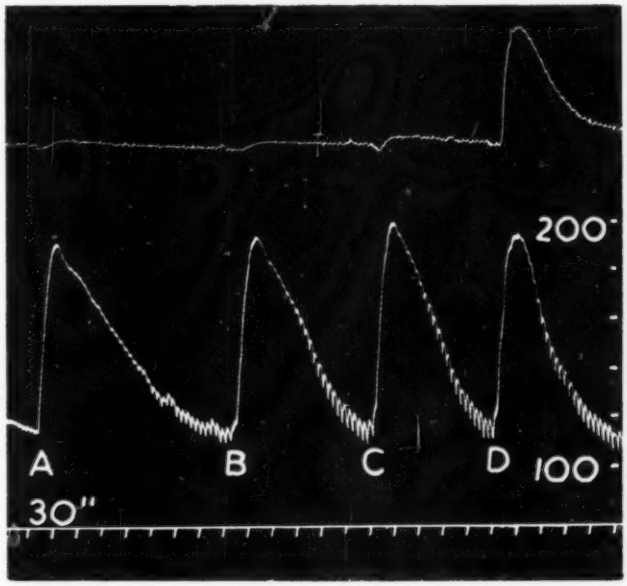

FIG. 4.—Same cat as in Figs. 2 and 3. Lergigan (2 mg./kg.) and atropine (0.5 mg./kg.) had now been given. A = 3 μg. *l-nor*adrenaline. B = 3 μg. *l-nor*adrenaline + 1 μg. histamine. C = 3 μg. *l-nor*adrenaline + 1 μg. acetylcholine. D = 7.5 μg. *l*-adrenaline.

Fig. 4 also reveals another interesting fact. Cocaine is well known to make the cat more sensitive to both adrenaline and *nor*adrenaline. The disadvantage of cocaine is that the normal nictitating membrane becomes sensitized to these substances in such a way that the difference between them becomes rather small, thereby reducing the possibility of using this organ for distinguishing between adrenaline and *nor*adrenaline. If, however, atropine is given the sensitivity of the membrane becomes altered, the action of adrenaline still being markedly potentiated whereas the sensitivity towards *nor*adrenaline is considerably decreased.

Spinal cats.—The effect of similar mixtures was tested on the blood pressure and the nictitating membrane of the spinal cat. In this preparation the admixture of small doses of histamine has a very marked effect on the pressor action of *nor*adrenaline. This is best illustrated in Fig. 5, in which it can be seen that 20 μg. *nor*adrenaline exerts a considerably stronger pressor effect than a mixture of 20 μg. *nor*adrenaline plus 1 μg. histamine, whereas the effect on the nictitating membrane is exactly the same (adrenals tied off). The same figure shows that if the histamine has been counteracted by giving an antihistamine the mixture becomes equiactive with pure *nor*adrenaline.

COMMENTS AND CONCLUSIONS

Adrenaline, *nor*adrenaline, histamine, and acetylcholine occur together in most tissues. When tissues are extracted it is natural that these substances may also occur in the extracts. When such extracts are tested biologically the effect of the pressor

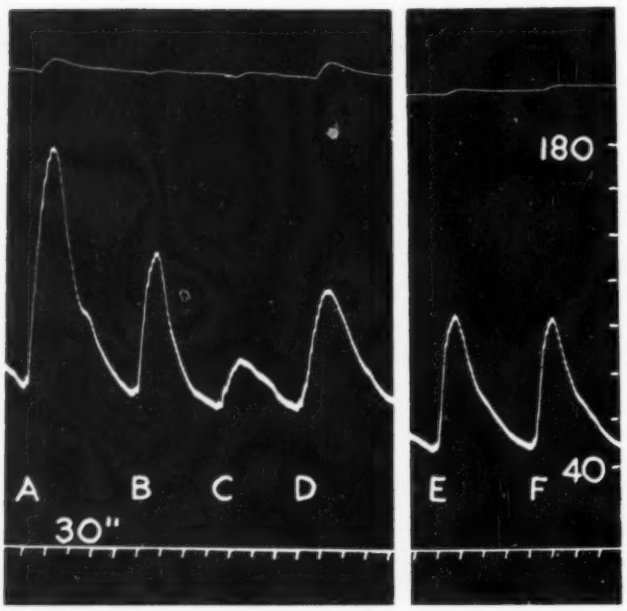

FIG. 5.—Spinal cat, adrenals removed. Normal nictitating membrane above, blood pressure below. A = 20 μg. *l-nor*adrenaline. B = 10 μg. *l-nor*adrenaline. C = 10 μg. *l-nor*adrenaline + 1 μg. histamine. D = 20 μg. *l-nor*adrenaline + 1 μg. histamine. Between D and E Lergigan (2 mg./kg.) was given. E = 10 μg. *l-nor*adrenaline + 1 μg. histamine. F = 10 μg. *l-nor*adrenaline.

substances may predominate to such an extent that the effect of the depressor substances is completely hidden.

When tissue extracts are tested on the cat's blood pressure and nictitating membrane with a view to estimating the amount of pressor substances, and simultaneously also the percentage of adrenaline and *nor*adrenaline, it must be remembered that contamination with even small amounts of histamine or acetylcholine may give rise to erroneous estimations. The reason for this is given in the present investigation.

1. The contaminating histamine and acetylcholine cause a release of adrenaline (or perhaps a mixture of adrenaline and *nor*adrenaline) from the suprarenals of the test animal itself.

2. These blood pressure lowering substances prevent the pressor substances from exerting their full action—they act as a braking mechanism.

The results of these two actions are the following. If adrenaline is released in the test animal the effects of this adrenaline will be added to the effects of the pressor substances in the extract, the effect of adrenaline being much stronger than *nor*adrenaline on the nictitating membrane (or on the non-pregnant uterus). The result of this adrenaline liberation will be interpreted in different ways, depending upon the nature of the pressor substance occurring in the extracts; three possibilities exist, viz., (*a*) if the extract contains *nor*adrenaline as the only pressor base the biological estimation may give the impression that there is in fact a mixture of adrenaline and *nor*adrenaline, (*b*) if the extract contains both adrenaline and *nor*adrenaline a false impression may be obtained about the percentage of each present, and (*c*) if the extract contains adrenaline as the only pressor base, estimation on the nictitating membrane may indicate a higher adrenaline content than that on the blood pressure, a result which might be interpreted as due to the presence of a pressor substance with a stronger effect on the nictitating membrane than adrenaline.

This release from the adrenals can, of course, be avoided if the adrenals are tied off. But even then contaminating histamine or acetylcholine may cause an error because of the second action mentioned above, viz., the braking mechanism. The pressor substances in the extracts cannot exert their full action on the blood pressure but will do so on the nictitating membrane or uterus, which are not affected by small doses of histamine or acetylcholine. The same arguments as those given above hold true even now. Let us assume that a tissue extract contains *nor*adrenaline and histamine and that 1 ml. of this extract, injected into the cat with adrenals tied off, is equipressor with 10 μg. *nor*adrenaline. On the nictitating membrane the extract exerts, however, a stronger contraction than the *nor*adrenaline. This finding suggests that the extract also contains adrenaline, whereas the real explanation is that 1 ml. of the extract actually contains 20 μg. *nor*adrenaline, the action of which was partly counteracted by the histamine on the blood pressure but not on the nictitating membrane. In other words, 1 ml. acted on the blood pressure like 10 μg. *nor*adrenaline, on the membrane it acted like 20 μg.

These errors exist when the biological estimation of amounts and percentages of adrenaline and *nor*adrenaline is performed on the same animal whether spinal or under chloralose. But even if separate preparations are used for these estimations, errors can still be made since the amount of pressor substances is still determined on the cat's blood pressure even if the percentage is estimated on isolated organs.

In order to avoid these erroneous estimations of pressor substances in extracts contaminated with histamine or acetylcholine the following precautions must be taken. The action of histamine and acetylcholine must be completely counteracted, which can be achieved by giving a suitable antihistamine and atropine. Furthermore, the adrenals should be tied off to exclude any possible liberation of pressor substances from them. The cat thus prepared is a very sensitive and reliable test object and can be used for the estimation of the total amount as well as percentage of adrenaline and *nor*adrenaline in a way analogous to the method described by Burn, Hutcheon, and Parker (1950).

A quite different way of avoiding the above-mentioned errors is to use extracts free from contaminating blood pressure lowering substances. Such an extraction method has recently been described by Euler (1948, 1949). In this method the pressor substances are adsorbed on to aluminium hydroxide, the blood pressure lowering substances remaining unadsorbed. This method of extraction has obviously a big advantage compared with other methods and should naturally be adopted when possible. Unfortunately it cannot always be used because the procedures involved take rather a long time.

Summary

1. Tissue extracts prepared for biological estimations of adrenaline and *nor*-adrenaline are often contaminated with histamine or acetylcholine. This admixture may cause a false impression of the amount and nature of the pressor substances, usually exaggerating the proportion of adrenaline.

2. Histamine and acetylcholine spoil the proper biological estimation of pressor substances in two ways:

(*a*) they release adrenaline from the adrenals of the test animal, and

(*b*) they prevent the pressor substances from exerting their full activity.

3. Special precautions must be taken when performing biological tests of such extracts.

4. A suitable preparation for this purpose is the cat under chloralose, treated with cocaine and given an antihistamine and atropine; the adrenals must also be tied off. If the blood pressure and the contractions of the normal nictitating membrane are recorded on this preparation adrenaline and *nor*adrenaline can be accurately estimated in spite of contaminating histamine or acetylcholine. The administration of atropine is essential for the discrimination between adrenaline and *nor*adrenaline.

5. If possible the extraction method described by Euler should be used. By this method the pressor substances are extracted but not the blood pressure lowering substances, the above-mentioned errors thus being avoided.

I am greatly indebted to Professor J. H. Burn for his great hospitality and for facilitating my work in this department.

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THE FORMATION *IN VIVO* OF *NOR*ADRENALINE FROM 3 : 4-DIHYDROXYPHENYLSERINE (*NOR*-ADRENALINE CARBOXYLIC ACID)

BY

CARL G. SCHMITERLÖW*

From the Department of Pharmacology, Oxford University

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The amino-acid 3 : 4-dihydroxyphenylserine was first described by Rosenmund and Dornsaft (1919), who suggested that it might be a precursor of adrenaline. During the extensive studies of enzymic formation of pressor amines carried out in this laboratory, Blaschko, Holton, and Sloane Stanley (1948) found that the decarboxylase present in *Streptococcus faecalis* R was able to decarboxylate dihydroxyphenylserine *in vitro*, the amine formed being *l-nor*adrenaline. They did not, however, obtain any evidence that the mammalian amino-acid decarboxylase, present, for example, in guinea-pig's kidney, was able to bring about this reaction. Recently Beyer (1950) observed that mammalian tissue extracts could in fact convert *nor*adrenaline carboxylic acid into a pressor substance with evolution of CO_2, and jointly with Blaschko, Burn, and Langemann (1950) he summarized evidence that the pressor substance was *nor*adrenaline. The work in this laboratory (Blaschko, Burn, and Langemann, 1950) showed that decarboxylation occurs, but only at a very slow rate and with fairly large amounts of tissue extract present, which was the reason why it had not been observed earlier. The amine formed exerted a strong pressor action in the spinal cat and at the same time only a weak action on the normal nictitating membrane and thus resembled *nor*adrenaline. Calculation showed that only the laevorotatory stereoisomer was produced.

Dopa-decarboxylase was originally described as an enzyme present in mammalian kidneys (Holtz, Heise, and Lüdtke, 1938), and, although it has later been shown to exist in other organs, renal tissue seems to be by far the most active source. Bing (1941) and Bing and Zucker (1941) showed that ischaemic cat's kidneys were able to produce a pressor substance, presumably hydroxytyramine, by decarboxylation of dihydroxyphenylalanine both in perfusion experiments and in experiments *in vivo*. Holtz, Credner, and Koepp (1942) made experiments on various animals (and man), and found that after both oral and parenteral administration of Dopa the urine contained hydroxytyramine.

From these considerations the question arose whether the administration of dihydroxyphenylserine to experimental animals would lead to an analogous output of *nor*adrenaline in the urine.

* With a grant from the Swedish Medical Research Council.

Methods

Rabbits were chosen as the most suitable animals. A good diuresis can easily be obtained after giving water through a stomach tube without an anaesthetic.

The animals were given 100 ml. tap water in order to start diuresis. The next day the same amount of water was given in the evening and the animals were put in metabolism cages. The urine produced was collected over-night in beakers in which 0.5 ml. of hydrochloric acid (conc. HCl diluted 1 : 1) was placed to keep the urine acid. These samples were collected in order to determine whether pressor substances were present in considerable amount in the normal urine of the rabbit.

The next evening the animals were given dihydroxyphenylserine intravenously, usually 5 mg. per kg. body weight. The amino-acid was dissolved in a few ml. of saline. Immediately after this they were again given 100 ml. tap water by stomach tube and put in the cages. The urine was collected over-night, acid being added as described above. The *p*H of the urine, thus collected, was usually about 3–4.

The dihydroxyphenylserine used was a sample of the material prepared by Dalgliesh and Mann (1947), previously used by Blaschko and his co-workers.

In a few experiments adrenalectomized rabbits were used in order to find out if the removal of the suprarenals interfered with the decarboxylation of dihydroxyphenylserine.

The amount of urine formed by the rabbits was usually well over 100 ml. In preliminary experiments it was found that this urine did not exert any significant action when tested on the cat's blood pressure and nictitating membrane. The urine was therefore concentrated by two methods.

The first method was as follows: Two volumes of alcohol were added to the acidified urine and the mixture was put in the refrigerator for two hours. It was then filtered on a Buchner funnel and the filtrate evaporated *in vacuo* to a small volume, 1 ml. of the final extract usually corresponding to 5 or 10 ml. of urine.

As it was soon found that these concentrates of urine contained blood pressure lowering substances in amounts large enough to interfere with the successful estimation of the *nor*adrenaline present, the method of adsorbing catechols described by Euler (1948, 1949), Euler and Hellner (1950), and Goodall (1950) was adopted in the following manner: Enough sulphuric acid was added to the urine to bring the *p*H to about 1. A 20 per cent (w/v) solution of $Al_2(SO_4)_3$ was added to the filtered urine in an amount equal to 2 per cent of the urine volume and also 1 ml. of BDH " universal indicator." From a burette 0.5 per cent (w/v) NaOH was added drop by drop, stirring continuously until a *p*H of 7.5 was reached, as shown by the colour change. The precipitate of aluminium hydroxide thus formed was then allowed to settle and collected on a Buchner funnel. The precipitate was washed three times with distilled water and then dissolved in a small volume (5–10 ml.) of 2N-H_2SO_4. To this solution 1 per cent (w/v) NaOH was added to bring the *p*H to about 3. Four volumes of a mixture containing equal parts of alcohol and acetone were then added and the solution left over-night in the refrigerator. The precipitated salts were filtered off and the filtrate evaporated to a small volume *in vacuo*.

For reasons which will be discussed later the majority of the extracts were tested on the blood pressure and the nictitating membrane of the cat under chloralose with the suprarenals tied off and after the intramuscular administration of cocaine (3 mg./kg.), an antihistamine (2 mg. Lergigan/kg.), and atropine (0.5 mg./kg.).

Results

Normal rabbit's urine.—The samples of normal urine treated with alcohol and concentrated were invariably found to cause a blood pressure fall when injected into the cat. Since Anrep, Ayadi, Barsoum, Smith, and Talaat (1944) have shown that

rabbit urine contains small amounts of free histamine it seemed probable that this fall was due to the presence of histamine. For this reason the antihistamine Lergigan, described by Halpern and Schmiterlöw (1951), was given in a dose of 2 mg./kg. After this the same samples still gave a slight drop in blood pressure although the effect of equiactive amounts of histamine was abolished. This small fall disappeared, however, when atropine was given, the extracts now having almost no effect on the blood pressure. Sometimes there was a very small rise, not amounting to more than a few mm. of Hg. It has been shown by several authors that normal urine from different species of animals contains pressor substances, but from these results the amount present in the concentrate from 10–20 ml. rabbit urine is negligible. In Fig. 1 is shown the effect of 1 ml. of normal urine concentrate, equivalent to 10 ml. of original urine, before and after the test animal (cat under chloralose) was treated with Lergigan and atropine. The figure shows that after the administration of the antihistamine the

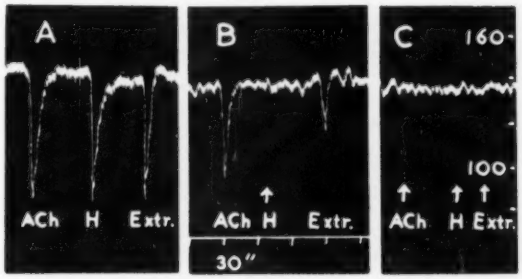

FIG. 1.—Cat; chloralose; blood pressure record. ACh = 1 μg. acetylcholine. H = 2 μg. histamine (base). Extr. = 1 ml. of urine extract (= 10 ml. of original urine) obtained by treating the urine with alcohol and subsequent concentration. (A) Before an antihistamine drug and atropine were given; (B) after 2 mg. of Lergigan per kg.; (C) after Lergigan and 0.5 mg. of atropine per kg.

effect of a dose of histamine, originally giving the same fall in blood pressure as 1 ml. of the urine concentrate, was now abolished, whereas the effect of an equiactive dose of acetylcholine was only diminished to a slight extent. After a further dose of atropine neither acetylcholine nor histamine nor the urine concentrate caused any effect on the blood pressure.

When the normal urine was treated by Euler's adsorption method the final concentrated extracts did not lower the blood pressure. This is in full accordance with the statement of Euler (1949) that this adsorption method gave " extracts with pure pressor activity." Evidently depressor substances are not adsorbed on the aluminium hydroxide. Neither did these extracts cause any significant rise of blood pressure (see Fig. 3, D).

Urine from rabbits given dihydroxyphenylserine.—When the rabbits received dihydroxyphenylserine intravenously their urine was found to contain a pressor principle.

At first the urine samples were treated with alcohol and concentrated. When these concentrates were injected into the chloralosed or spinal cat the effect on the blood pressure was an initial fall, followed by a slight secondary rise. After the administration of Lergigan and atropine to the test animal the urine samples no longer produced a fall in blood pressure but a conspicuous rise, which was enhanced by cocaine. Fig. 2 shows the difference in effect of the urine samples before and after the administration of the antihistamine.

The blood pressure rise thus caused by the urine samples was matched against *nor*adrenaline. It was found that 10 ml. of urine (= 1 ml. of concentrate) gave an effect equal to that of about 2–3 μg. *l-nor*adrenaline.

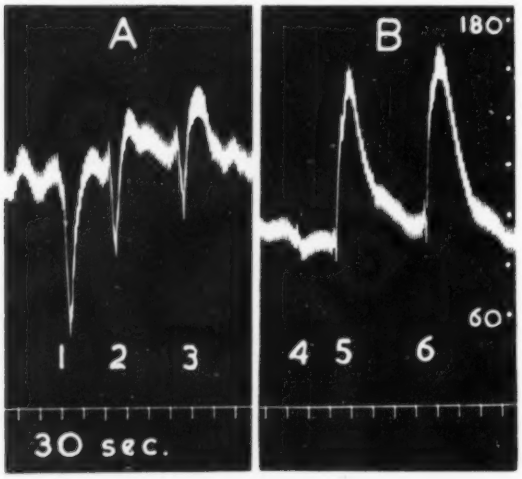

FIG. 2.—Cat; chloralose; blood pressure record. 1 and 4 = 2 μg. histamine (base). 2 and 5 = 1 ml. of concentrated urine (= 5 ml. of original urine) collected from rabbit given 5 mg. dihydroxyphenylserine per kg. intravenously. Urine treated with alcohol and concentrated. 3 and 6 = a similar sample from another animal. (A) Before and (B) after the administration of 2 mg. Lergigan per kg.

Absence of adrenaline.—As the amino-acid injected was *nor*adrenaline carboxylic acid, the pressor effect was most probably due to *nor*adrenaline, derived from the amino-acid through decarboxylation. The possibility exists, however, that adrenaline may also be formed *in vivo* from this compound, which would lead to the output of a mixture of adrenaline and *nor*adrenaline.

One method of estimating these substances in a mixture has been described by Burn, Hutcheon, and Parker (1950); in this a spinal cat is used and the blood pressure and the contraction of the normal nictitating membrane recorded. The method, however, requires a concentration of about 10 μg./ml. of the two amines, which is much greater than was present in the urine concentrates. The cat under chloralose was therefore used instead, the sensitivity of which is much higher especially when cocaine has been administered (3 mg./kg. by intramuscular injection). Cocaine, however, diminishes the difference between the effect of *nor*adrenaline and of adrenaline on the nictitating membrane, but the difference can be brought out again by giving atropine (0.5 mg./kg.). This method of testing is discussed elsewhere (Schmiterlöw, 1951). It was found satisfactory when applied to urine concentrates prepared by the adsorption method. When alcoholic extracts were examined it was necessary to tie off the adrenals and to give Lergigan and atropine as well as cocaine.

When the urine extracts were now tested it was found that their effect on the nictitating membrane corresponded exactly to the effect caused by equipressor doses of *l-nor*adrenaline. There was no sign of an admixture of adrenaline.

Yield of nor*adrenaline.*—In the early experiments varying doses of dihydroxyphenylserine were injected, 15, 10, and 5 mg. per kg., but no significant relationship between the injected dose and the output of *nor*adrenaline was observed. For this reason and because of shortage of the amino-acid, the lowest dose, 5 mg. per kg., was used throughout the rest of the experiments. In all, 22 experiments were done with this

dose in 12 rabbits. In all the experiments the urine contained a pressor constituent which behaved like pure *nor*adrenaline. The diuresis of the rabbits varied, the lowest output of urine during approximately fifteen hours (from the evening till next morning) being 75 ml., the highest 245 ml. The calculated average output of *nor*adrenaline was found to be 0.34 $\pm$ 0.03 (standard deviation $\pm$ 0.13) μg. per ml. of urine. If the total output of *nor*adrenaline was compared with the amount of dihydroxyphenylserine given (5 mg. per kg.) the yield of *nor*adrenaline from the amino-acid was on an average 0.39 $\pm$ 0.02 (standard deviation $\pm$ 0.11) per cent.

In Fig. 3 is shown the effect of one of the extracts (adsorption method) on the blood pressure and nictitating membrane in comparison with the effect of *nor*adrenaline and adrenaline. The effect of normal urine from the same rabbit is also shown.

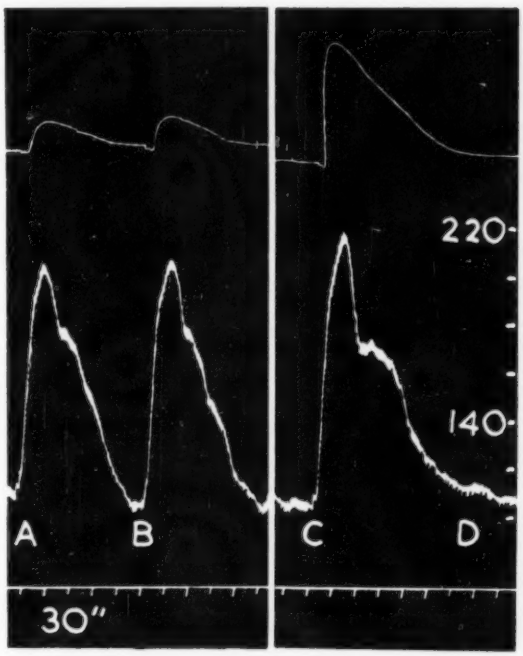

FIG. 3.—Cat; chloralose; normal nictitating membrane above, blood pressure below. Cocaine (3 mg. per kg.) and atropine (0.5 mg. per kg.) given. A = 1 ml. of urine concentrate (= 10 ml. of original urine) obtained by the adsorption method; urine from rabbit which had been given 5 mg. dihydroxyphenylserine per kg. B = 3 μg. *l-nor*adrenaline. C = 7.5 μg. *l*-adrenaline. D = 1 ml. of urine concentrate (= 10 ml. of urine) obtained by the adsorption method; normal urine from the same rabbit as in A.

In two rabbits under urethane the effect of the intravenous injection of dihydroxyphenylserine on the blood pressure was recorded. No alteration in the blood pressure could be observed. If the amino-acid was injected into the cat there was no rise in blood pressure even if a dose of 2 mg. was given.

Presence of adrenal glands.—The appearance of *nor*adrenaline in the urine from rabbits given dihydroxyphenylserine was not influenced by the removal of the suprarenals, the output still being approximately the same as before. This was so whether the experiment was performed the day after the operation or eight days later.

Adrenaline carboxylic acid.—In four rabbits the effect of N-methyl-β-(3 : 4-dihydroxyphenyl) serine (= adrenaline carboxylic acid) was also tested, the same amount of this amino-acid being used as of the *nor*adrenaline carboxylic acid. The

urine from these rabbits, treated according to Euler's adsorption method, gave a slight rise in blood pressure (Fig. 4, C). This effect is, however, of little significance, since the compound used was contaminated with adrenaline. It is shown in Fig. 4 that the injection of 2 mg. of this amino-acid sample caused a rise in blood pressure equal to that caused by 5 μg. adrenaline, but a greater contraction of the nictitating membrane.

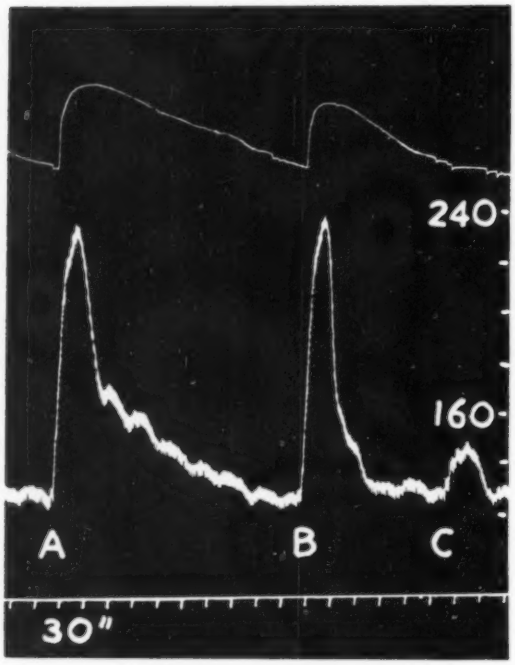

FIG. 4.—Cat; chloralose; normal nictitating membrane above, blood pressure below. Cocaine (3 mg. per kg.) and atropine (0.5 mg. per kg.) were given. A = 2 mg. adrenaline carboxylic acid. B = 5 μg. *l*-adrenaline. C = 1 ml. of urine concentrate (= 10 ml. of original urine) obtained by the adsorption method; urine from rabbit which had been given 5 mg. adrenaline carboxylic acid per kg. intravenously.

COMMENTS

The present investigation has shown that the urine from rabbits which have been given dihydroxyphenylserine (*nor*adrenaline carboxylic acid) contains *nor*adrenaline. This observation is an extension of the recent *in vitro* findings that this amino-acid can serve as a substrate for the mammalian amino-acid decarboxylase (Beyer, 1950; Beyer, Blaschko, Burn, and Langemann, 1950; Blaschko, Burn, and Langemann, 1950). It is now evident that this decarboxylation in fact also takes place *in vivo*.

During recent years extensive studies have been carried out on the presence and physiological role of *nor*adrenaline in various tissues and body fluids. Little is known, however, about the biosynthesis of this substance. In a recent review Blaschko (1950) gives the different pathways along which adrenaline and *nor*adrenaline may be formed in the body. It is tempting to assume, in the light of both *in vitro* and *in vivo* studies now available, that *nor*adrenaline carboxylic acid may serve as a precursor to *nor*-adrenaline. The main objection against such an assumption is, of course, that this amino-acid is at present a synthetic compound which has not yet been shown to exist in the body as a natural constituent. It seems to be of great interest to study this

question. Another objection could be that the amount of *nor*adrenaline formed from dihydroxyphenylserine *in vivo* in the present experiments was very small, the yield of *nor*adrenaline being only about 0.3 per cent of the injected amount of amino-acid. This figure is considerably less than that found in earlier experiments *in vitro*. It must be remembered, however, that the experiments *in vitro* were carried out under anaerobic conditions which prevented the further oxidation of the *nor*adrenaline formed. Even if a fairly large amount of *nor*adrenaline was in fact produced from the amino-acid *in vivo* it could hardly be expected that all this would appear in the urine, since it must be presumed that most of the amine would be rapidly oxidized in the body. For this reason little significance should be attributed to the quantitative estimation of the *nor*adrenaline recovered from the urine, but emphasis should rather be put on the fact that it occurs at all as a result of a decarboxylation process. Further investigations may show whether the output increases when the kidneys are rendered ischaemic or other precautions are taken to diminish oxidative processes to a minimum.

The results of testing the urine samples on the blood pressure and nictitating membrane of the cat under chloralose indicated that the pressor substance found was *nor*adrenaline only; they did not indicate whether the amine formed was the laevorotatory compound or the racemic form. In the *in vitro* experiments only *l-nor*adrenaline was formed (Blaschko, Burn, and Langemann, 1950). It is interesting to note that there was no evidence of the formation of adrenaline from the dihydroxyphenylserine, especially in view of the finding by Bülbring (1949) that suprarenal tissue is able to methylate *nor*adrenaline. Adrenaline if formed might have been destroyed before excretion.

When adrenaline carboxylic acid was given instead of *nor*adrenaline carboxylic acid the urine samples only caused a very slight rise in blood pressure. This rise was explained by a contamination of the amino-acid used with adrenaline. It was shown by Blaschko, Burn, and Langemann (1950) that adrenaline carboxylic acid does not serve as a substrate for amino-acid decarboxylase *in vitro* and it was therefore not to be expected that the administration of this amino-acid would lead to an output of adrenaline.

Summary

1. When dihydroxyphenylserine, which is also known as *nor*adrenaline carboxylic acid, was administered to rabbits by intravenous injection in a dose of 5 mg. per kg., varying amounts of *nor*adrenaline were found in the urine.

2. The experiments were performed by making the injection when diuresis was established. The urine was collected, concentrated, and freed from depressor substances by Euler's method. The extract was tested on the blood pressure and nictitating membrane of the cat under chloralose. The test made it possible to show that the active material was *nor*adrenaline alone and that no adrenaline was present.

3. Normal rabbit urine extracted in this manner contained no pressor substances in the doses used.

4. The transformation still occurred in animals after adrenalectomy.

5. The injection of the corresponding adrenaline carboxylic acid was not followed by the appearance of any pressor substance in the urine.

6. The possibility that *nor*adrenaline carboxylic acid is a natural precursor to *nor*adrenaline has been recently supported by experiments *in vitro*. The present investigation shows that this decarboxylation occurs *in vivo*, and this finding throws some light on the biosynthesis of *nor*adrenaline.

It is a great pleasure for me to express to Professor J. H. Burn my sincere gratitude for his great hospitality and never-failing support during my stay in his department. I am also greatly indebted to Dr. H. Blaschko and Dr. E. Bülbring for their continual help and advice during this investigation.

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THE WALKER CARCINOMA 256 IN THE SCREENING OF TUMOUR INHIBITORS

BY

A. L. WALPOLE

From Imperial Chemical Industries, Ltd., Biological Laboratories, Wilmslow, Manchester

(Received November 22, 1950)

In the early stages of the examination of compounds as potential therapeutic agents for malignant disease it is customary to study their action upon malignant tumours, spontaneous or transplanted, in the smaller laboratory animals, rats or mice. Various tumours and conditions of test have been employed and various criteria of activity adopted. For the preliminary screening of compounds of several series we have used as test object the Walker carcinoma 256. The nature of the compounds and the results obtained with them have been the subject of a preliminary communication (Rose, Hendry, and Walpole, 1950) and will be published in greater detail in the near future; the object of the present paper is to describe our routine screening procedure and some experiments upon the growth of the tumour under various experimental conditions which provide a background for the critical evaluation of those results.

The earlier history of the Walker tumour has been described by Earle (1935). It was obtained by us from Professor Haddow and has been maintained for several years in these laboratories by serial subcutaneous implantation at eight to twelve days' intervals in albino rats of a heterozygous stock in breeding here. Both stock rats and those upon experiment are housed upon sawdust in galvanized wire mesh cages and are allowed to feed *ad libitum* upon a composite diet in pellet form with tap water available at all times; the diet, which is obtainable from Scottish Agricultural Industries, Limited, has the following percentage composition by weight:

Fine wheat middlings	19.2	White fish meal (60% protein)	4.7
Ground wheat	19.2	Dried skimmed milk	*7.0
Sussex ground oats	19.2	Dried yeast	1.2
Ground barley	9.5	Sodium chloride	0.5
Ground maize	9.5	Cod liver oil	0.5
Meat and bone meal	9.5		

* Increased in later batches to 14%

Our standard procedure for the detection of tumour growth inhibitory activity is similar to that described by Haddow and Robinson (1937) and is essentially as follows:

A rat carrying an actively growing implant, eight to twelve days old, is killed by fracture of the spinal cord and the tumour exposed under aseptic conditions. Fragments cut from the healthy peripheral part and as nearly equal in size (200–300 mg.) as can be judged by the eye are implanted by trochar and cannula, subcutaneously, in the right flanks of a number of rats, each 90 to 120 g. in weight. Each rat receives a single implant and the skin wound is closed with a Michel clip. The rats are then separated into groups of from ten to fifteen animals, matched in respect of sex distribution and mean body weight.

Where it is found necessary to use more than one tumour to provide implants for all the animals in any one experiment, the implants from each tumour are distributed equally

between the several groups. Compounds under test are usually given by intraperitoneal injection, started on the day following implantation and continued daily, Sundays excepted, for the first ten to twelve days of experiment. Occasionally only one dose is given and that within the first few days of the operation. The treated rats are weighed daily and the doses adjusted for body weight changes. The control animals remain untreated, since we have found that the vehicles which we use in making up compounds for injection have no effect upon the growth of the tumour in the quantities employed. On the fourteenth or fifteenth day of experiment, the day of implantation being taken as day 0, all the animals surviving the experiment are weighed and killed and the tumours dissected out, cut in several planes, " blotted " on absorbent wool, and weighed.

Early in the course of our experiments it became apparent that although conditions had been standardized as far as was practicable the tumours which developed in rats implanted with tissue from any one tumour and treated in a manner identical in all other respects varied considerably in size at fourteen or fifteen days. This applied equally to control animals and those which had been dosed with a compound. Examination of the results from a large number of experiments showed that the distribution of tumour weights within groups of rats treated alike was rather unusual. For control animals it was bimodal with one peak between 0 and 2 g. and the other at about 26 g. (Fig. 1).

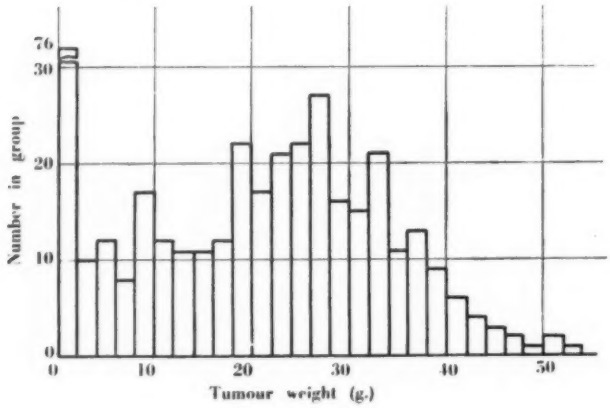

FIG. 1.—Distribution of tumour weights in control animals.

The proportion of rats contributing to the lower peak was about one-third of the total, including animals in which the tumour did not " take," while the distribution of the remaining weights was almost normal. This finding was consistent with the supposition that about one-third of our stock were highly resistant to the growth of the tumour while the remainder were considerably more susceptible, giving, when untreated, tumours weighing up to 50 g. or more.

With these considerations in mind several different methods were examined in order to define the most sensitive means of assessing the statistical significance of the effect of any treatment. The most sensitive method found was to select from each group of treated or control animals the upper half of the distribution of tumour weights (i.e., the n heaviest tumours out of any group of $2n$), to determine the mean, M_{5c}, of each selection, and to assess the statistical significance of the difference in the normal way, assuming that the weights chosen are complete samples. The method is equivalent to selecting that half of all the rats which are most susceptible to tumour growth.

The percentage inhibition of tumour growth, I, we define by the formula:

$$I = \frac{(M_{50} \text{ controls} - M_{50} \text{ treated})}{(M_{50} \text{ controls})} \times 100$$

where M_{50} is the mean referred to above.

The choice of method for assessing the significance of the effect of a treatment is governed by the nature of the distribution of the tumour weights. If this changed, as it might well do if, for example, rats of a different stock were used, then the method recommended might no longer be the most sensitive. The growth of the Walker tumour in rats from our colony has of late been more uniform than in our early experiments and the proportion of highly resistant animals seems to be decreasing. It would be rash to assume that this desirable tendency is permanent, but in several experiments we should be justified in utilizing the whole of the tumour weights in both control and treated groups in calculating the magnitude of the effect and its significance.

Sources of variation in tumour growth rate

The variation encountered in the weights of tumours from identically treated animals under the conditions of our test might arise from (*a*) variation in the rats and/or (*b*) factors dependent upon the technique, such, for example, as differences in the size or condition of the implants. In an experiment designed to separate and estimate the contribution from each of these sources, portions of tissues from two tumours, A and B, were implanted subcutaneously in the right and left flanks respectively of each of 20 rats (10 males and 10 females). The weights of the two tumours in each of the animals fourteen days later are shown in Table I. A marked tendency for both implants in any one rat to grow relatively poorly or relatively well is apparent. The analysis of the variations as given by Dr. O. L. Davies is set out in Table II.

TABLE I

TUMOUR WEIGHTS AT 14 DAYS FROM IMPLANTS OF TUMOURS A AND B IN RIGHT AND LEFT FLANKS, RESPECTIVELY, OF 20 RATS

	Tumour weights in grammes																			Total
Right flank .. (tumour A) ..	19	18	16	16	14	13	12.5	12	11	11	10	10	10	10	4	4	4	3	0.7	0.3	198.5
Left flank (tumour B) ..	16	16	12	12	16	14	14	8.5	16.5	17	16	16	13	8	3	3	3	4	1.1	2	211.1

TABLE II

ANALYSIS OF VARIATION OF FIGURES SHOWN IN TABLE I

Source of variation	Sum of squares	Degrees of freedom	Variance
Original tumours	3.95	1	3.95
Rats	1,131.40	19	59.55
Remainder	107.44	19	5.65
Total:	1,242.79	39	

The difference in the growth of the implants from tumours A and B considered as a whole is not significant. The remainder variation, which includes that dependent upon the technique, is small compared with the variation between rats. The analysed variances are:

Remainder	5.65
Due to rats (59.55—5.65)/2 ..	27.00

It is concluded that there would be no appreciable gain in precision if more than one tumour were implanted into each rat.

The term " variation between rats " as used above covers not only genetically determined differences in resistance or susceptibility to the growth of the tumour but all differences, e.g., of initial age and body weight, of body growth rate, and of nutritional status, such as might conceivably affect the rate of growth of the implants. Experiments to determine the influence of such variables have so far yielded the results described below.

TABLE III

EFFECT OF INITIAL WEIGHT OF RATS UPON WEIGHT OF WALKER TUMOUR IMPLANTS AT 14 DAYS (CONVENTIONS AS IN TEXT)

	Group I			Group II			Group III		
	Initial wt. of rat (g.)		Tumour weight (g.)	Initial wt. of rat (g.)		Tumour weight (g.)	Initial wt. of rat (g.)		Tumour weight (g.)
	♂	♀		♂	♀		♂	♀	
		50	36.0		111	33.0		185	43.0
	57		28.0	108		32.0	258		36.5
	53		27.0	99		32.0	228		34.0
	53		23.0		109	30.0	257		30.0
	47		22.0	102		27.5		177	29.0
	46		21.0		98	27.5		208	29.0
	53		21.0	97		26.5	272		24.0
		47	21.0		94	23.75	264		21.0
		50	20.5	103		22.5	281		18.0
	52		20.0	110		20.5	284		17.5
		48	20.0	97		17.5		208	15.5
	58		16.5	99		13.0	344		12.5
		50	16.0		95	12.75	241		11.5
		47	15.5		91	12.5	314		10.0
	51		8.75		107	12.0		236	2.0
	45		5.0		96	8.5		204	1.0
		49	2.0		99	1.5		220	0.5
	56		0.0	95		1.0	269		0.0
	49		0.0	97		0.0	200		0.0
		43	0.0	99		0.0		224	0.0
		48	0.0	102		0.0		187	0.0
		48	0.0		93	0.0		206	0.0
		49	0.0		97	0.0		197	0.0
		49	0.0		99	0.0		217	0.0
Means	49.9			99.9			236.7		
M_{100}			13.47			14.75			13.96
M_{50}			23.0			25.48			25.83
M_{50} (♂ s)			23.6			26.83			27.25
M_{50} (♀ s)			21.5			23.25			19.9

Influence of initial age and body weight of rats

An experiment was carried out in which the Walker tumour was implanted into rats of three groups differing widely in body weight. Each group contained 24 animals, 12 males and 12 females. The rats in the first group each weighed between 43 and 58 g. (mean 49.9 g.), those in the second group between 91 and 110 g. (mean 99.9 g.), and in the third between 177 and 344 g. (mean 236.7 g.). Four tumours were used to provide the implants and the same number of rats in each group received a single implant from each of these. The rats were killed and the tumours weighed fourteen days later. The tumour weights, arranged in each group in descending order of magnitude, and the corresponding weights of the rats on the day of implantation are shown in Table III.

The mean tumour weights, M_{100}, in the three groups were 13.47, 14.75, and 13.96 g. respectively, and do not differ significantly from one another in spite of the enormous differences between the mean body weights of the rats. The same applies to the mean weights of the upper 50 per cent of tumours, M_{50} in the three groups being 23.0, 25.48, and 25.83 g. respectively. It is concluded that no significant part of the extreme variation encountered in the tumour weights can be attributed to differences in the initial rat weights.

It will be noted that in each group tumour growth appeared somewhat more vigorous in the males than in the females. This may be correlated with the greater rate of growth of male rats as compared with females.

The foregoing considerations suggest very strongly that the variations in tumour growth rate are dependent largely upon genetically determined differences in susceptibility to tumour growth. We have considered attempting to breed by selection a strain of rat of enhanced susceptibility with a view to obtaining more uniform tumour growth, but so far have not done so.

The influence of nutritional status

An interesting early study of the influence of nutritional conditions upon body growth and tumour growth in tumour-bearing animals is that of Moreschi (1909). He transplanted " sarcoma 7 " into mice weighing about 11 g. and then separated the animals into four groups. In the first three each mouse was given daily 1, 1.5, and 2 g. of food respectively, while those in the fourth group were allowed to feed freely. The tumours grew in proportion to the amount of food the mice received. The gross weight gain of the animals was also directly proportional to this. On the other hand the net mouse weight (gross weight − tumour weight) fell in each group, the decrease being small but definite in the animals feeding *ad libitum* and more marked in the undernourished groups. The increase in gross weight of the well-nourished mice was entirely due to the more rapid growth of the tumours in this group. It was noted that the undernourished mice often died at a later juncture than those feeding freely. A similar experiment with mice weighing over 20 g. initially yielded essentially the same result. Experiments of a similar kind with various tumours in rats or mice have been reported by Haaland (1907), Jensen (1909), Rous (1911, 1914), Sweet, Corson-White, and Saxon (1913), and show that with many tumours at least undernourishment of the host leads to reduction in the rate of tumour growth.

The effect of hypophysectomy as well as of underfeeding upon the growth of the Walker tumour has been studied by McEuen and Thomson (1933). In their experi-

ments a reduction in tumour growth rate was produced by hypophysectomy comparable with that obtained by partial starvation of the tumour-bearing rats. It is clear, however, from the work of Elson and Haddow (1947) that a reduction in body growth rate in animals carrying this tumour does not invariably lead to a reduction in tumour growth rate. They implanted the tumour into rats maintained on diets containing 20, 10, and 5 per cent of protein respectively and followed body and tumour growth for periods up to thirteen days. The mean net body weight of the rats receiving the 20 per cent protein diet increased during this period while that of the rats on the two other diets fell, but no significant difference was observed in the rate of growth of the tumour in the three groups. Some observations of Bischoff and Maxwell (1931) and of Bischoff, Maxwell, and Ullman (1931) are of interest in this connexion. They applied *x*-rays to the pituitaries of rats bearing sarcoma 10 or the Hyde rat carcinoma, with the result that, if the irradiation was sufficient to inhibit the growth of the animals, tumour growth was inhibited also. On the other hand they observed no inhibition of tumour growth when body growth was checked by treating the tumour-bearing animals with various toxic substances or by partially starving them.

We have carried out experiments upon the influence of underfeeding upon the growth of the Walker tumour in which the conditions approximate more closely to those of our standard test than any described in the literature.

In a typical experiment the tumour was implanted into rats in four groups each comprising six males and six females. The mean body weight at implantation in each of the four groups was approximately the same and lay between 96.5 and 98.2 g. Food was supplied in the form of our standard pellets broken down to granules of such size that they passed through a ten-mesh screen but were retained by a twenty-mesh screen. It had been observed that rats would pick such granules individually from a food pot and consume them at leisure with

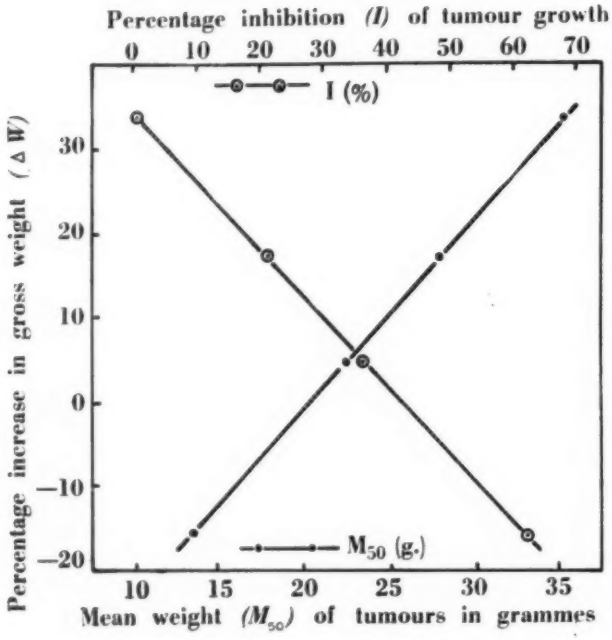

FIG. 2.—Effect of dietary restriction upon tumour growth and gross weight gain in rats bearing the Walker tumour. ●——●, plot of M_{50} against ΔW. ○——○, plot of I against ΔW.

very little wastage. Where, as in this experiment, several animals were kept in a cage with the supply of food restricted, its use in this form ensured that each animal secured its fair share, and as wastage was minimal the daily food intake could be closely controlled. The rats in group I were allowed to feed freely, those in groups II, III, and IV were each allowed 7, 5, and 3 g. of food respectively per day. Restriction of the diet was instituted on the day of implantation of the tumour and continued until the surviving animals were killed and the tumours weighed fourteen days later. Tap water was available at all times.

The results are shown in Table IV. In Fig. 2 the mean percentage increase in gross weight (ΔW) of the surviving animals in each group is plotted against the mean weight (M_{50}) of the heavier 50 per cent of the tumours, and against the percentage

TABLE IV

EFFECT OF UNDERFEEDING UPON GROWTH OF THE WALKER TUMOUR. (CONVENTIONS AS IN TEXT.)
RAT WEIGHTS MARKED * EXCLUDED FROM CALCULATION OF MEANS

	Group I			Group II			Group III			Group IV		
	Rats fed *ad libitum*			Rats fed 7 g. per day			Rats fed 5 g. per day			Rats fed 3 g. per day		
	Gross wt. (g.)		Tumour wt. (g.)	Gross wt. (g.)		Tumour wt. (g.)	Gross wt. (g.)		Tumour wt. (g.)	Gross wt. (g.)		Tumour wt. (g.)
	Initial	Final		Initial	Final		Initial	Final		Initial	Final	
	92	122	40.0	114	136	32	110	113	25.0	98	86	14.0
	101	147	39.0	102	131	30	78	111	24.0	110	94	13.5
	93	133	37.0	102	120	27	104	108	22.5	105	83	13.0
	109	144	33.5	74	100	27	103	107	22.0	95	92	13.0
	103	125	32.0	91	100	26	98	96	21.0	98	80	10.0
	104	142	29.5	100	120	25	97	98	19.0	89	80	9.0
	90	121	28.0	90	116	24	99	112	19.0	104	84	8.0
	106	125	28.0	104	124	24	100	104	19.0	94	70	4.0
	102	160	26.0	108	114	23	107	92	17.0	99*	Dead	—
	82	116	25.5	104	116	22	97	108	15.0	100*	,,	—
	103	127	25.0	96	93	22	98	94	13.0	82*	,,	—
	84	102	22.0	93	111	22	88*	Dead	—	94*	,,	—
Mean	97.42	130.3		98.17	115.1		99.2	103.9		99.13	83.63	
ΔW		33.7			17.2			4.75			−15.6	
M_{50}			35.2			27.8			22.58			13.4
I			—			21			35.8			61.9

inhibition (I) of tumour growth. It is clear that underfeeding may produce a considerable inhibition of the growth of this tumour, and it appears that over a considerable range M_{50} is proportional to the increase in gross weight of the animals.

In our standard screening experiments all the animals are allowed free access to a diet of fixed composition, but the consumption or assimilation of the food or both undoubtedly varies from group to group under the influence of the compounds given. Any treatment which significantly reduces body growth rate, either by reducing food consumption, impairing the digestive and assimilative functions, or by more obscure and subtle means, is likely to reduce the growth rate of the tumour, although, in view of the observations of Elson and Haddow (1947), it need not necessarily do so. When the administration of a compound is found to lead to inhibition of tumour growth it is desirable to know to what extent that inhibition is merely a manifestation of some such

general " nutritional " or " toxic " effect, and to what extent it is in any sense directed preferentially towards the tumour. From Fig. 2 we can roughly estimate the probable upper limit of the tumour inhibition due to general " toxicity " associated with a given reduction in gross weight gain. It appears that underfeeding at the level required to prevent any increase in mean gross weight inhibits tumour growth by about 40 per cent. This has been several times confirmed. In many of our experiments the dosage of compounds under test has been so adjusted that the mean gross weight of the treated tumour-bearing rats has been held roughly constant throughout. We conclude as a first approximation that any compound which in these circumstances produces inhibition in excess of this figure must act in some way other than by a non-specific " toxic " effect.

Some examples of the various types of response encountered in routine experiments are shown in Table V. The majority of compounds examined produce no greater inhibition of tumour growth than can be accounted for on the basis of non-specific toxicity, as indicated by the effect on gross weight gain. Stilboestrol is of this type, and

TABLE V

THE EFFECT OF SOME COMPOUNDS UPON GROSS WEIGHT GAIN AND THE GROWTH OF THE WALKER TUMOUR IN RATS

Compound	Total dose per 100 g. rat, given over 10–12 days	ΔW		M_{50}		I
		Controls	Treated	Controls	Treated	
Stilboestrol	225 mg. i.p. in oil	31.8	7.5	20.9	18.0	14.0
N-Benzoylethylene-imine	10 mg. i.p. in oil	38.3	13.9	35.3	34.7	1.7
8-Azaguanine	200 mg. i.p. in aqueous suspension	36.0	1.0	29.3	12.9	56.0
Trimethylolmelamine	250 mg. i.p. in aqueous suspension	31.2	14.9	30.3	0.5	98.0
Tris-ethylene-imino-triazine	0.14 mg. i.v. in aqueous solution	28.5	14.3	37.2	2.0	95.0

a result obtained with large doses is shown. A small number of substances has been found which reduce the gross weight gain of tumour-bearing animals without producing any appreciable inhibition of tumour growth. This result is analogous to that obtained by Elson and Haddow (1947) by reducing dietary protein. One such substance is N-benzoylethylene-imine. The compound 8-azaguanine (" guanazolo ") has been reported by Kidder, Dewey, Parks, and Woodside (1949) to retard the development of several mouse tumours. This is one of a number of substances which only inhibit the growth of the Walker tumour to a slightly greater extent than can be attributed to non-specific toxicity. Compounds which are of more particular interest as potential therapeutic agents, however, are those with a clearly specific action upon the tumour. Trimethylolmelamine and 2 : 4 : 6-tris-ethylene-imino-1 : 3 : 5-triazine (T.E.T.) are examples of this type.

Summary

A technique for the screening of compounds for tumour growth inhibitory activity using the Walker carcinoma 256 in rats is described. The influence of various factors upon the growth of this tumour has been studied. The tumours which develop

in fourteen days from subcutaneous implants in albino rats of heterozygous stock vary considerably in size from rat to rat; this variation is mainly due to inherent differences in susceptibility to tumour growth.

Tumour growth is virtually independent of the age and weight of the rats at implantation; it is rather more vigorous in males than in females and is reduced by underfeeding. The inhibition of tumour growth produced by underfeeding is proportional to the reduction in gross weight gain of the tumour-bearing rats. Underfeeding at the level required to prevent any increase in gross weight inhibits tumour growth by about 40 per cent.

Compounds which interfere with body growth may be expected to inhibit tumour growth as a result of their general " toxic " action. This inhibition is unlikely to exceed that which would result from underfeeding at a comparable level. Any inhibition which a compound may produce in excess of that which can be attributed to interference with body growth must be due to factors other than non-specific " toxicity."

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PHARMACOLOGICAL STUDY OF INJECTED CHOLINESTERASE

BY

I. T. BECK

From the Department of Pharmacology, Faculty of Medicine, McGill University, Montreal, P.Q., Canada

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The aim of producing muscular relaxation during abdominal operations has been achieved with curare and other neuromuscular blocking agents, but unfortunately the use of such drugs is not always without risk, and the reversal of their action by neostigmine cannot be relied upon.

The possibility was considered that cholinesterase in excess might inhibit the transmission of nervous impulses to the muscle. Its use would have the advantage that its paralytic action could be immediately nullified by the administration of anticholinesterase drugs. To the best of the author's knowledge, no experiments have been carried out to explore this possibility.

If it could be proved that injected cholinesterase blocks neuromuscular transmission, another proof might also be established in favour of the theory of chemical transmission between nerve and muscle, although failure to produce such evidence would not invalidate this theory.

Most of the work published on cholinesterase concerns the inhibition of its action by different drugs, and very few studies deal with the augmentation of the acetylcholine-hydrolysing capacity of the organism, which should follow the injection of cholinesterase. Mendel and Hawkins (1943) reported on the effects of injection into rats of horse serum pseudocholinesterase, dog pancreas pseudocholinesterase, and true cholinesterase prepared from the electric organ of the torpedo. They observed that the chromodacryorrhoea produced by subcutaneous injections of acetylcholine could be prevented if horse serum or dog pancreas pseudocholinesterase was injected intravenously before the administration of acetylcholine. Furthermore, they were able to show that these enzymes were not only capable of annulling the effects of injected acetylcholine but also that liberated at nerve synapses. They reached this conclusion on the basis that these enzymes interrupted the pupillary light reflexes. They obtained similar results in a few experiments with the true cholinesterase prepared from the electric organ of the torpedo.

Recently Lesuk (1949) prepared a very potent true cholinesterase from bovine red blood cells. No pharmacological work on its action is known to the author apart from two recent short notes by Koppanyi and Karczmar (1948, 1949); their results will be referred to later. In this study Lesuk's preparation will be referred to as the

red cell enzyme.* Cholinesterase, if meant generally, is referred to as such throughout this work.

METHODS

Preparation of solutions of red cell enzyme.—The dried material was dissolved in sterile 2 per cent (w/v) gelatin in distilled water to make a solution of 1,000 units per ml. Withdrawals were carried out under sterile conditions. The container was kept in a refrigerator. No decrease in activity was observed during storage over six weeks. The activity of the preparation was in reasonable agreement with that stated by the manufacturer.

Determination of cholinesterase.—The acetylcholine employed in all experiments was the chloride obtained from Hoffman-LaRoche. An amount of 0.5 ml. of the enzyme, or the enzyme-containing blood or tissue extract, was put into the side arm of a Warburg vessel and the substrate (acetylcholine, final concentration 0.01 M) into the main compartment. The Ringer solution in which the acetylcholine was dissolved had the same composition as that described by Birkhaüser (1940). The total volume of fluid in the Warburg vessel was 2 ml. The quantity of red cell enzyme or cholinesterase which liberated 1 μl. of CO_2 (equivalent to 8 μg. acetylcholine base) in one minute was considered as one unit. Readings were made every 5 minutes for 30 minutes when the cholinesterase activity of blood, and every 10 minutes for 1 hour when that of muscle extracts, were determined. Each result given is the mean of two parallel determinations.

Choice of animals.—In most experiments male and female rats weighing about 300 g. were used. Rats were chosen for the experiments because of their relatively small size and the resultant economy of enzyme. Care was taken in each series of experiments that about half the animals were males and half females in view of the report by Sawyer and Everett (1947) of a sex difference of serum cholinesterase levels. In some experiments cats were used. The animals were anaesthetized with 70 mg. chloralose per kg. intravenously after an ether induction. As an anticoagulant 1 mg. sodium heparin per kg. was used.

Electrical stimulation.—In all the experiments where nerves were stimulated, square wave stimuli of 3 milliseconds' duration were applied, though frequency duration and amplitude varied in different kinds of experiments.

RESULTS

Blood cholinesterase activity after intravenous injection of 1,000 *units of red cell enzyme*

It is known that injected horse serum pseudocholinesterase remains for several days, and dog pancreas pseudocholinesterase only for about one hour, in the blood of rats (Mendel and Hawkins, 1943).

About 1 ml. of blood was withdrawn by intracardiac puncture from rats weighing about 300 g. Of this blood 0.5 ml. was added to 1.5 ml. distilled water containing 66 μg. heparin per ml. to prevent coagulation. One thousand units of red cell enzyme were then injected into a cannula tied in the jugular vein and washed in with 0.5 ml. saline solution; 2, 5, or 10 minutes after the injection 1 ml. of blood was withdrawn by cardiac puncture and diluted in the same way as the first sample. Not more than two blood samples (before and after injection of red cell enzyme) were removed from the same animal in order to avoid excessive disturbance of the blood volume. Determinations of cholinesterase content were carried out by the manometric method described above, 0.5 ml. of the diluted blood being used; as

* The author is indebted to Dr. M. L. Tainter, director of Winthrop-Stearns, Inc., for a supply of Lesuk's preparation.

0.125 ml. of blood was in the vessel, the results were multiplied by 8 in order to obtain cholinesterase values for 1 ml. of blood.

After the injection of 1,000 units of red cell enzyme, the cholinesterase activity of whole blood is increased for the first two minutes but rapidly falls off and may even be decreased ten minutes after injection (Table I). The average increase in the total

TABLE I

No.	Weight and sex of rat	Cholinesterase activity before injection of 1,000 units red cell enzyme. Units/ml. blood	Time between injection and withdrawal of second blood sample	Cholinesterase activity after injection of 1,000 units red cell enzyme. Units/ml. blood	Difference in units/ml. blood before and after injection of enzyme
1	300 g. ♂	16.4		77.9	+ 61.5
2	330 g. ♂	16.8	2	33.1	+ 16.3
3	270 g. ♀	25.4	minutes	65.0	+ 39.6
4	240 g. ♀	15.8		55.7	+ 39.4
Mean		18.6		57.9	+ 39.2 ± 9.2*
5	260 g. ♀	21.0		17.6	− 3.4
6	270 g. ♀	19.2	5	23.1	+ 3.9
7	295 g. ♂	25.5	minutes	25.4	− 0.1
Mean		21.9		22.0	+ 0.1 ± 2.1*
8	270 g. ♂	16.1		15.5	− 0.6
9	290 g. ♀	25.9	10	24.4	− 1.5
10	320 g. ♂	20.5	minutes	17.5	− 3.0
Mean		20.8		19.1	− 1.7 ± 0.7*

* Standard error.

blood cholinesterase activity of 39.2 units/ml. indicates a total increase of 800 units if it is assumed that a 300-g. rat has a blood volume of about 20 ml. This means that two minutes after the injection practically all the injected red cell enzyme was still in the circulating blood. On the other hand, five minutes after the injection, nearly all the injected enzyme had disappeared from the blood.

The rather big error in the results at five minutes may be explained by the fact that five minutes is the approximate limit of the increased cholinesterase activity of the blood. In some animals there is still a slight increase; in others the activity has returned to normal or even a negative value may be found. It may seem paradoxical that after injection of red cell enzyme the total cholinesterase activity should decrease (rats Nos. 5, 8, 9, and 10 in Table I), but this is probably due to dilution of the blood. This dilution is due (1) to the loss of the 1 ml. blood sample drawn for the first analysis, which represents approximately 5 per cent of the total blood volume and is presumably replaced by extracellular fluids poorer in cholinesterase than blood; (2) to the 1.5 to 2 ml. fluid injected with the red cell enzyme (1 ml. containing 1,000 units and 0.5 to 1 ml. saline to wash it in through the cannula). These two factors together account for 12 to 15 per cent dilution, which covers very well the " loss " of cholinesterase, once the originally injected enzyme has disappeared.

Muscle cholinesterase activity after intravenous injection of 1,000 *units of red cell enzyme*

It was necessary to know whether injected enzyme reaches the muscle if an attempt to evaluate its action on neuromuscular conduction was to be made.

The skin over both gastrocnemius muscles of male and female anaesthetized rats was incised, and after an interval of ten minutes the right gastrocnemius was cut out with as little trauma as possible. Then 1,000 units of red cell enzyme were injected intravenously, and after 2, 5, or 10 minutes the left muscle was removed. Extracts from these were prepared as follows: about 1 to 2 g. muscle was washed for 30 seconds with Ringer solution (Birkhaüser, 1940), dried with filter paper, and dissected free of connective tissue. After being weighed it was cut into small pieces, placed on a wire gauze, and again washed with Ringer solution; 6 ml. of Ringer solution were now added for each gramme of tissue, and the muscle was ground with glass powder in a mortar (a 1-g. muscle piece was ground for 6 min. and 2 g. for 12 min.). The ground material was filtered through two layers of muslin. For each determination 0.5 ml. (= 83.3 mg. muscle) of the filtrate was used.

Because of the limited quantity of red cell enzyme available the author was not able to carry out many experiments, but the results in Table II probably indicate the trend of events, at any rate. It will be seen that the cholinesterase activity of muscle is increased at least during the first ten minutes. This is not due to operative shock, because if no enzyme is injected the second muscle tends to have a lower activity than the first muscle (animals Nos. 7, 8, and 9). The relatively high value two minutes after the injection is probably due to the high enzyme content of the blood at this time (Table II).

TABLE II

No.	Weight and sex of rat	Cholinesterase activity before injection of 1,000 units red cell enzyme. Units/g. muscle	Time between injection and taking the second muscle sample for test	Cholinesterase activity after injection of 1,000 units red cell enzyme. Units/g. muscle	Difference in units/g. muscle before and after injection of enzyme
1	275 g. ♂	2.51	2 minutes	4.33	+1.82
2	330 g. ♂	2.67	5 minutes	3.24	+ 0.57
3	310 g. ♂	2.06		2.54	+ 0.48
4	220 g. ♀	5.81		7.05	+ 1.24
Mean		3.51		4.27	+ 0.76 ± 0.24*
5	330 g. ♂	3.12	10 minutes	3.57	+ 0.45
6	300 g. ♀	4.81		5.71	+ 0.90
Mean		3.96		4.64	+ 0.67 ± 0.22*
7	310 g. ♂	5.24	No enzyme injected; 2nd muscle excised 7.5 min. after the 1st	4.60	− 0.64
8	230 g. ♀	4.40		4.35	− 0.05
9	250 g. ♂	3.96		3.50	− 0.46
Mean		4.53		4.15	− 0.38 ± 0.17*

* Standard error.

Though the total cholinesterase activity in the muscle is increased after injection of red cell enzyme the results do not discriminate between an increase taking place in the muscle fibres themselves and that in the extracellular fluid.

Acetylcholine hydrolysis in vivo *by red cell enzyme as measured by the effect of injected acetylcholine and of vagus stimulation on the blood pressure of rats*

The carotid artery of anaesthetized rats was cannulated and the blood pressure measured by a mercury manometer with the help of the amplifier described by Bayo, Wilbrandt, and Lauener (1948) for amplifying intestinal pressure changes. With this method only relative changes of blood pressure are determined. In some experiments the apparatus was calibrated in terms of absolute pressures. Injections, followed by saline, were made into the jugular vein. In other experiments the vagus was prepared and stimulated at 60 per sec. for 10 sec. with a strength sufficient to produce a marked fall in blood pressure.

After establishing the hypotensive reaction by different doses of intravenously injected acetylcholine, 1,000 to 2,000 units of red cell enzyme were administered intravenously, and the acetylcholine test was repeated several times.

It was found, as was to be expected from the blood cholinesterase activity values, that after the injection of red cell enzyme (Table I) the action of injected acetylcholine was reduced only during the first few minutes after the injection of the enzyme, but not after five minutes (Fig. 1). In none of the experiments was the acetylcholine action completely abolished.

The hypertension after acetylcholine injection shown in Fig. 1, which precedes the typical depressor effect, is due to the amount of injected liquid. The acetylcholine

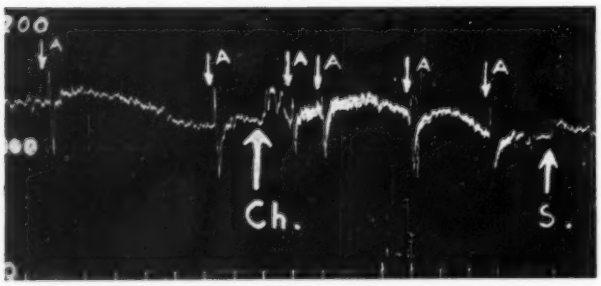

FIG. 1.—(Exp. 28.) Rat. ♂ 290 g. Chloralose. B.P. record. Time in minutes. A = 0.01 μg. acetylcholine i.v. Ch = 1,000 units red cell enzyme followed by 1 ml. saline. S = 1 ml. saline.

was washed into the jugular vein through the cannula with 1 ml. saline, which is about 5 per cent of the blood volume. The hypertension after injection of enzyme (Ch) is probably due to the same cause. It may be seen that saline alone can have the same effect (S). In animals which are less sensitive to an increase of blood volume this hypertension does not take place (Fig. 2*a*), so that the conclusion seems justified that red cell enzyme does not affect the blood pressure. This is in agreement with Sarkar, Maitra, and Ghosh (1942), who showed that even large amounts of cobra-venom cholinesterase do not affect the blood pressure and respiration of the rabbit. They observed an increase of beat amplitude on the toad's heart. Schachter (1945), on the other hand, found that cholinesterase raised the blood pressure of shocked dogs.

The increase of oscillations shown in Fig. 1, two minutes after injection of enzyme, may be due to the increased blood volume or superimposed respiration, but not to the red cell enzyme itself. This phenomenon was not found in other experiments (see Figs. 2*a* and 2*b*).

In Figs. 2*a* and 2*b* it may be seen that red cell enzyme is totally ineffective in decreasing the effects of vagus stimulation on the heart, whether the nerve be stimulated immediately (2*b*) or 5 to 45 min. after injection (2*a*). (These results

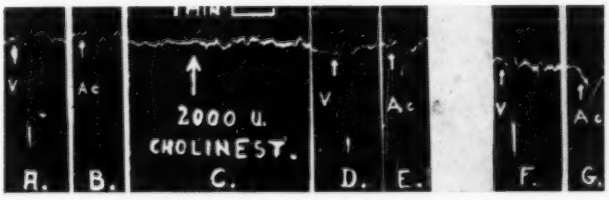

FIG. 2*a*.—(Exp. 6.) Rat. ♀ 330 g. Chloralose. Blood pressure. Drum rate in section C. *V* = Vagus stimulation: 60 per sec., for 10 sec., strength 1 volt. *Ac* = Acetylcholine chloride: 0.1 μg. intravenously. (A) and (B) before injection of cholinesterase. (C) 2,000 units red cell enzyme intravenously. (D) 5 min., (E) 8 min., (F) 45 min., and (G) 48 min., after the injection in (C).

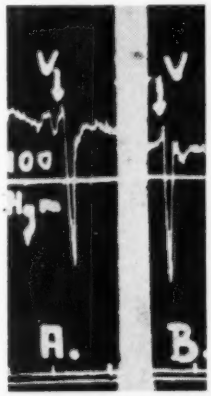

FIG. 2*b*.—(Exp. 30.) Rat. ♂ 290 g. Chloralose. Blood pressure. Time in minutes. *V* = Vagus stimulation: 60 per sec. for 10 sec., strength 7 volts. (A) Before injection of cholinesterase. (B) 2 min. after the injection of 1,000 units red cell enzyme.

contradict those of Koppanyi and Karczmar (1948), but are in accordance with later work of the same authors (1949).) The explanation of this will be discussed in connexion with neuromuscular transmission in striated muscle.

The action of red cell enzyme on neuromuscular transmission in vivo

Two different methods have been employed:

(*a*) The sciatic nerve of anaesthetized rats was prepared and the Achilles tendon freed and tied to one arm of an isometric torsion-spring myograph. The length of this arm was 1.5 cm. The other arm, 22.5 cm. long, formed the writing lever. Calibration was done with weights. The tibia was held in a clamp in order to immobilize the hind limb, with the nerve resting on silver wire electrodes; submaximal or supramaximal stimuli were applied at a rate of 2 per min. Injections of 1,000 to 2,000 units of red cell enzyme and of neostigmine sulphate were made through a cannula tied into the external jugular vein.

The twitch tension of the gastrocnemius did not change after 1,000 or 2,000 units of intravenously injected red cell enzyme. In order to be sure that the preparation had not deteriorated, and that other substances were still able to produce their effect, 50 μg. neostigmine sulphate per kg. was injected intravenously at the end of every experiment: it produced a prompt increase in twitch tension (Fig. 3).

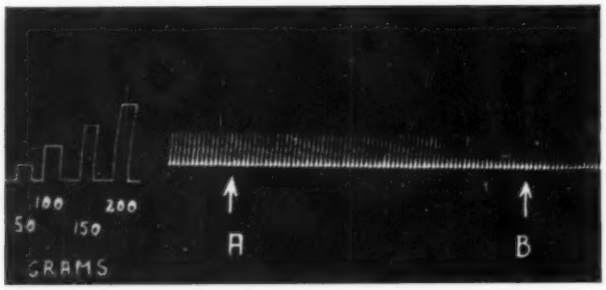

FIG. 3.—(Exp. 16.) Rat. ♀ 290 g. Chloralose. Isometric recording of the gastrocnemius. Submaximal stimulation of the sciatic nerve. One stimulus every 30 sec. Strength 7 volts. At A, 2,000 units red cell enzyme. At B, 33 min. later 50 μg. neostigmine sulphate intravenously.

(*b*) In order to increase the possibility of the enzyme reaching the endplates it was injected directly into the artery supplying the cat's tibialis anterior muscle and the isometric contractions of the muscle were recorded. In three experiments Brown's method (1938) was used and in one the enzyme was injected into the femoral artery, all the branches of the latter except that going to the tibialis anterior muscle having been tied. Stimulation at the rate of 6 per min. was applied in all four experiments to the peripheral end of the tibialis nerve; in two of supramaximal and in two of submaximal strength. With this technique a relatively high concentration of enzyme reached the muscle in a short time, and, if the enzyme were able to reach the endplate, a greater quantity should have done so than in the previous experiments. One to two thousand units of enzyme were injected in one and a half seconds. In none of the four experiments did any change in the twitch tension occur after the injection of the enzyme. One of the records is reproduced in Fig. 4.

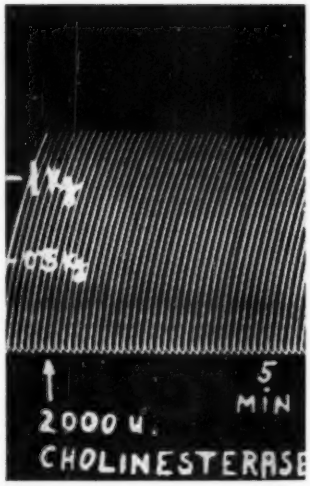

FIG. 4.—(Exp. 25.) Cat. ♂ 3.35 kg. Chloralose. Isometric recording of the tibialis anterior muscle. Supramaximal stimulation of the tibialis nerve, once every 10 sec. Strength 2 volts. At arrow, 2,000 units red cell enzyme. Close arterial injection; duration of injection 1.5 sec.

Action of red cell enzyme on neuromuscular transmission in vitro

In order to increase further the possibility of the enzyme reaching the muscular endplate two different isolated preparations were studied.

(*a*) *Isolated sciatic gastrocnemius preparation of the frog.*—The sciatic nerve and the gastrocnemius were prepared by the usual technique. The external fascia of the gastrocnemius was clipped open to allow easier penetration of the enzyme to the neuromuscular junction. The gastrocnemius was fixed at the knee in a 50-ml. bath containing Ringer solution, the composition of which was as follows: NaCl = 0.65 g.; KCl = 0.02 g.; $CaCl_2$ (anhydr.)

= 0.02 g.; 98 ml. distilled water; 2 ml. of phosphate solution (2.86 g. Na_2HPO_4 in 80.6 ml. 0.1 N-HCl and 19.4 ml. distilled water, *p*H = 7.1).

The contractions were recorded isometrically. The sciatic nerve was stimulated with single shocks twice per minute at supra- or sub-maximal strength. After stabilization of contraction had occurred, different quantities of red cell enzyme were added to the bath and their effects on the contractions observed.

(*b*) *Isolated phrenic nerve-diaphragm preparation of rat.*—For this experiment a preparation described by Bülbring (1946) was used. The stimulation of the phrenic nerve was 1 per sec. or 1 per 10 sec., supra- or sub-maximal, with the electrodes described by Bülbring. The bath contained 50 ml. of modified Tyrode solution aerated with 95% O_2 + 5% CO_2 (Taugner and Fleckenstein, 1950). Contractions are more stable and steadier in this fluid than in normal Tyrode. The isotonic contractions were recorded with a well-balanced light frog heart lever. The contractions before and after addition of red cell enzyme to the bath were observed.

Though the concentration of red cell enzyme in the experiments of both series was very high (20 to 40 units/ml.), no changes in contractions occurred after its addition to the bath (Fig. 5).

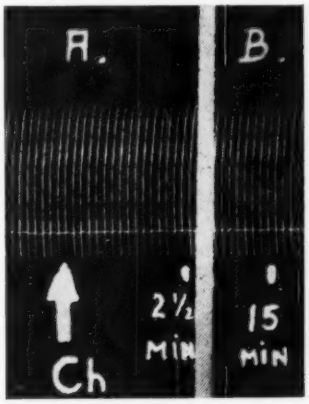

FIG. 5.—(Exp. 32.) Isotonic recording of the rat phrenic nerve-diaphragm preparation *in vitro*. Muscle bath containing 50 ml. modified Tyrode solution at 37° C. Supramaximal stimulation of the phrenic nerve once every 10 sec. Strength 5 volts. (A) *Ch* = 1,000 units red cell enzyme added to the bath. (B) 15 min. after addition of enzyme to the bath.

The action of red cell enzyme on the muscular contractions caused by close arterial injections of acetylcholine

Two experiments were performed with the method described by Brown (1938). In order to avoid the muscarinic effects of acetylcholine the cats were injected with 0.5 mg. atropine per kg.

In one experiment 10 μg. and in the other 20 μg. acetylcholine in 0.2 ml. saline were injected into the prepared tibialis artery of the cat. Intravenous injections of red cell enzyme did not affect the tension developed significantly (Fig. 6) despite the large amount of enzyme injected (9,000 units in one experiment and 10,000 in the other).

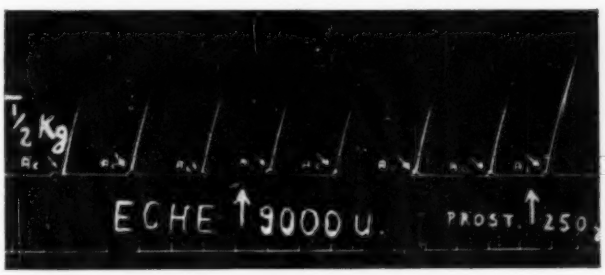

FIG. 6.—(Exp. 36.) Cat. ♀ 2.05 kg. Chloralose. Isometric recording of the tibialis anterior muscle. Atropine sulphate 0.5 mg./kg. i.v. Time in minutes. Ac = close arterial injections of 20 μg. acetylcholine chloride. ECHE = intravenous injection of 9,000 units red cell enzyme. Prost = intravenous injection of 250 μg. neostigmine sulphate.

In order to check the responsiveness of the muscle 250 μg. neostigmine sulphate were injected intravenously at the end of the experiments and the expected increase in tension of the contraction occurred.

The influence of red cell enzyme on chromodacryorrhoea induced by acetylcholine injections

It was of interest to know whether the experiments of Mendel and Hawkins (1943) could be repeated with this preparation of cholinesterase. The short duration of increased esterase activity in blood obtained after the injection of the red cell enzyme decreased this possibility since, according to the authors cited, cholinesterases which remain for a longer time in the blood counteract for a longer period the " bloody tears " effect of acetylcholine than those which remain for only a short time. For this reason the red cell enzyme could not be expected to cause more than a retardation of the appearance or a diminution of the abundance of the chromodacryorrhoea.

The experiments were carried out with the technique of Mendel and Hawkins; 1 mg. acetylcholine chloride was injected subcutaneously into rats weighing about 50 g. and the chromodacryorrhoea was observed by touching the eye with the edge of a filter paper. The coloration of the filter paper and the size of the drop after contact with the eye served as a measure of the acetylcholine action. This measurement was repeated at different intervals one to twenty minutes after the injection of acetylcholine, depending on the amount of chromodacryorrhoea. Five animals were tested without previous injection of enzyme; five others two minutes after injection of the enzyme. After one week a cross-over test was carried out. The red cell enzyme (37.5 to 1,000 units) was injected intravenously or intracardially.

It was observed that the chromodacryorrhoea produced by the subcutaneous injection of 1 mg. acetylcholine generally appeared in the first minute and reached its maximum about five minutes after the injection. Its usual duration was fifteen minutes, though occasionally it lasted longer.

In three animals out of ten the chromodacryorrhoea appeared to be slighter if the acetylcholine was injected after the red cell enzyme, but the response appeared within the normal time limit. In two animals it appeared to be greater when acetylcholine was injected after the enzyme, and in five other animals the injection of enzyme did not affect the course of chromodacryorrhoea produced by acetylcholine. Red cell enzyme seems, therefore, to have no effect upon this phenomenon.

No change in pupil diameter was caused by the red cell enzyme nor was the response to light modified.

Discussion

The rapid disappearance of the injected red cell enzyme from the blood stream is puzzling. Is it destroyed or inactivated, does it simply diffuse into the tissue spaces, does some special organ store it or does rapid excretion cause this quick disappearance? If it is destroyed the destruction does not occur in the blood; this is shown by the fact that, although all determinations of blood cholinesterase were done several hours after the blood sample had been taken, yet, as may be seen in Table I, when the blood was withdrawn two minutes after the injection, about 80 per cent of the total amount of injected enzyme was recovered; if the blood had continued to circulate, the total amount would have disappeared within five minutes. Diffusion cannot account for a

complete disappearance of the enzyme from the blood. The relatively small increase in muscle cholinesterase at a time when no increased activity in the blood remains removes the possibility of equal distribution in the body by diffusion. Had the injected cholinesterase been distributed equally throughout the extracellular fluid, or throughout the whole of the body water, the increase in muscle cholinesterase should have been of the order of about 3 units per g. muscle; instead, the mean increase observed was only 0.76 units per g. muscle. An appreciable excretion of red cell enzyme in the urine or faeces in so short a time is improbable. The only remaining possibility is inactivation of the enzyme or its accumulation by a special tissue. Koppanyi and Karczmar (1948) state that the liver is probably involved in the removal of the enzyme from the blood, but the experiments they mention indicate only that the disappearance is due chiefly to some process which occurs below the diaphragm.

During the short period in which the blood cholinesterase is increased the injection of acetylcholine produced a subnormal effect on the blood pressure. Evidently a portion is hydrolysed by the circulating enzyme.

The period in which the enzyme is effective against acetylcholine injections was much shorter in the present experiments (maximum 5 min.) than in those recently reported by Koppanyi and Karczmar (1948) (" less than 30 minutes ").

The negative nature of the results of the experiments in which the vagus, the sciatic of rats, and the tibialis anterior of the cat were stimulated, and of the experiments with the isolated gastrocnemius and diaphragm preparations, requires consideration. The most obvious explanation in all these experiments is the possible failure of the enzyme, injected into the general circulation, into the artery supplying the tibialis muscle, or applied directly to muscle and nerve, to reach the point essential for the elicitation of an effect. The conditions for penetration seem especially favourable in the diaphragm preparation; the muscle is very thin and the concentration of enzyme in the bath does not diminish as in the experiments in which the enzyme was injected intravascularly. It may be that some penetration did occur but that the excess of enzyme at the vital points is normally so high that the increases in concentration which may have occurred were too small to be detectable. It is known from the experiments of Eccles, Katz, and Kuffler (1941), Eccles and Kuffler (1941), and Kuffler (1942) that in skeletal muscle the endplate potential must be reduced to one-third of its original value before neuromuscular conduction fails appreciably. A study of the endplate potential changes after injection of the enzyme might throw some light upon the extent to which penetration actually occurs. The fact that even " close " injections of acetylcholine (into the tibialis artery) produced the same twitch of the tibialis muscle regardless of whether enzyme was injected or not indicates that the injection did not raise the enzyme concentration appreciably at the point essential for its action. A calculation based upon the extent to which the enzyme concentration of muscle is increased by the injection of 1,000 units of red cell enzyme in 300-g. rats shows that the increase in the entire tibialis anterior muscle of the cat is sufficient to hydrolyse only 0.3 μg. acetylcholine per quarter second. Since an injection of 10 to 20 μg. acetylcholine was made in a fraction of a second there could not have been enough destruction to affect its action.

These negative results are reminiscent of the persistent lack of response that Felix (1938) and Best and McHenry (1940) found on injection of their highly active histaminase preparation. Only Karady and Brown (1939) have been able to find that

injected histaminase counteracted histamine and anaphylactic shock, but Knoll (1940) and Youngner, Freedmann, and Nungster (1941) were unable to confirm their results.

Summary

1. The effects of intravenously injected bovine erythrocyte true cholinesterase (red cell enzyme) were studied.

2. The cholinesterase level in blood of 300 g. male and female rats is increased in the first two minutes and returns to normal five minutes after the intravenous injection of 1,000 units of red cell enzyme.

3. The muscle cholinesterase activity is increased for two to ten minutes or longer.

4. The hypotensive action of intravenously injected acetylcholine on the rat's blood pressure is partly reduced, but not abolished, immediately after the intravenous injection of 1,000 units of red cell enzyme. No reduction in the hypotensive effect of acetylcholine occurs five minutes after injection of the enzyme.

5. The blood pressure level of rats is not affected by the intravenous injection of 1,000 units of red cell enzyme.

6. The effect of vagus stimulation on the blood pressure of rats is not affected by the intravenous injection of 1,000 units of red cell enzyme.

7. Red cell enzyme injections do not influence neuromuscular transmission *in vivo* in the sciatic gastrocnemius preparation of the rat or in the tibialis anterior of the cat.

8. Red cell enzyme does not influence neuromuscular transmission in the isolated sciatic-gastrocnemius preparation of the frog or in the isolated phrenic-diaphragm preparation of the rat.

9. The twitch produced by close intra-arterial injection of acetylcholine is not affected by the administration of red cell enzyme.

10. The chromodacryorrhoea in rats produced by subcutaneous injection of acetylcholine is not affected by this preparation of cholinesterase, nor does it dilate the pupils of rats.

The author is indebted to Dr. A. S. V. Burgen for friendly discussions.

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PARALYSIS OF AUTONOMIC GANGLIA BY METHONIUM SALTS

BY

W. D. M. PATON AND E. J. ZAIMIS

From the National Institute for Medical Research, Mill Hill, London, N.W.7

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The production of ganglionic block by the lower members of the polymethylene bistrimethylammonium series was briefly described in earlier papers (Paton and Zaimis, 1948, 1949a, b). The experiments, which are now reported in full, were designed to analyse the mechanism by which these compounds paralyse autonomic function and to determine how far such ganglionic blockade accounts for the pharmacological properties of the drugs. We have found that the compounds interfere with transmission only at the synapse itself, that they cause block without any previous excitation, and that they do not interfere with the release of acetylcholine in the ganglion. Further, they are free from other pharmacological actions, in particular from curare-like, muscarine-like, or histamine-liberating activity. They constitute, indeed, the most specific ganglionic inhibitors so far described.

Certain members (the butane, pentane, and hexane derivatives) have been submitted to clinical trial, and have received the approved names of tetramethonium, pentamethonium, and hexamethonium respectively. These names will be used in this paper; the other members of the series will be referred to by the number of carbon atoms in the polymethylene chain.

METHODS

Cats anaesthetized with chloralose (80 mg./kg.) were used for most experiments. In experiments on the superior cervical ganglion, the contractions of the nictitating membrane were recorded on a smoked drum. These contractions were evoked by the application of maximal " square-wave " electrical stimuli, of 0.5 msec. duration, to the cut peripheral stump of the cervical sympathetic, divided and separated from the vagus in the neck, or to the postganglionic trunk after dissection of the ganglion. Perfusion of the ganglion was by the technique of Kibjakow (1933) and Feldberg and Gaddum (1934), the perfusion fluid being warmed to the cat's body temperature by its passage through a tube passed into the stomach of the cat and emerging from a small cut in the oesophagus, close to the ganglion (MacIntosh, 1949). The perfusion fluid was Locke's solution containing 5×10^{-6} eserine. The acetylcholine in the perfusate was assayed on the blood-pressure of an eviscerate, eserinized cat.

Cat's blood-pressure was recorded in the usual way by means of a siliconed cannula containing a little 1 per cent heparin, inserted into the carotid artery. For studying the effects of vagal stimulation, the left or right vagus nerve was divided in the neck, and the peripheral end was separated from the sympathetic and excited electrically. To record intestinal activity in the cat, a length of small intestine, about 6 cm. long, was selected and glass cannulae inserted at each end, tying them in by ligatures round the submucosa only; after the intestine

had been washed out, it was filled with saline, one cannula was occluded, and the other cannula was connected to a sensitive bellows recorder. Experiments on the peristaltic reflex of the rabbit's ileum were made by the method of Trendelenburg (1917).

Intravenous injections were made through a glass cannula tied into the jugular or femoral vein.

Vasodilatation in the rabbit's ear was recorded by means of Hatfield's (1950) heat-flow meter; this consists of a tellurium disk 1 cm. diameter, with copper gauze welded to either side. When placed on a surface losing or absorbing heat, a potential difference, easily recorded by a galvanometer, is set up between the two thermo-junctions so formed, such that for a heat loss of 100 kg. cal./sq. m./hour an electromotive force of approximately 70 microvolts is obtained. The disk is stuck to the surface of the ear by a layer of cellulose tape.

Methonium salts, tetraethylammonium, and tetramethylammonium were all given as iodides, and nicotine as tartrate. Doses are all given in terms of the salts.

Results

Action on cat's superior cervical ganglion

If the cut peripheral end of the cervical sympathetic in the cat is stimulated electrically at a frequency of 10/sec., a well-sustained contraction of the nictitating membrane can be recorded. This is depressed or abolished by the injection intravenously of substances which block transmission in the superior cervical ganglion. Such a relaxation can be produced by the members of the methonium series from C3 to C10, but with various degrees of activity; pentamethonium and hexamethonium are particularly effective, and doses of 0.02 mg./kg. injected intravenously sometimes produce a detectable effect. Fig. 1 illustrates this action for hexamethonium in a dose of 0.23 mg./kg.

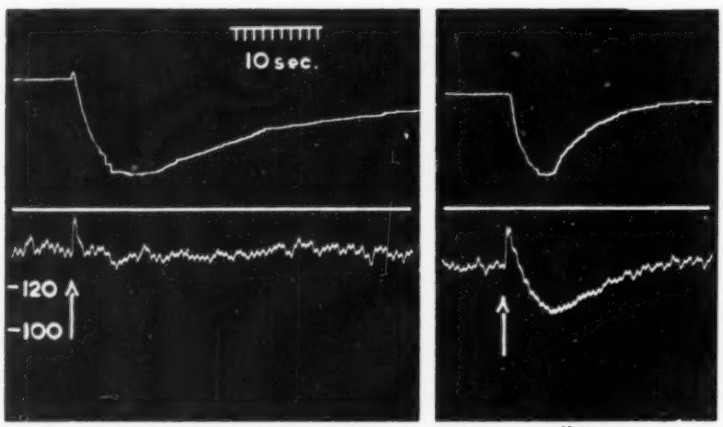

FIG. 1.—Cat, chloralose. Contraction of nictitating membrane to preganglionic excitation at 10 shocks/sec. (above) and blood-pressure (below). (*a*) At arrow, 0.23 mg. hexamethonium iodide per kg. intravenously; (*b*) at arrow, 1.5 mg. tetra ethyl ammonium iodide per kg. intravenously.

The sensitivity of the ganglion depends on the nature and duration of the preganglionic excitation. For instance, if stimulation at a rate of 10/sec. is maintained for 15–20 min., the proportionate reduction of the height of contraction of the nictitating membrane by a given dose of pentamethonium or hexamethonium may be 20–30 per cent greater at the end than at the beginning of excitation. Similarly, a ganglion which receives continous stimulation is more sensitive to these drugs than

one which receives brief periods of stimulation at intervals. The experiments of Fig. 1 and 2 exemplify this. In Fig. 1, the continuously excited ganglion was 75 per cent blocked (as measured by the response of the membrane) by 0.23 mg. hexamethonium per kg.; whereas in Fig. 2, with intermittent excitation, twice this dose of hexamethonium only reduced the tension developed after 10 sec. of stimulation by about 15 per cent. Fig. 2 also shows how the ganglion becomes more sensitive

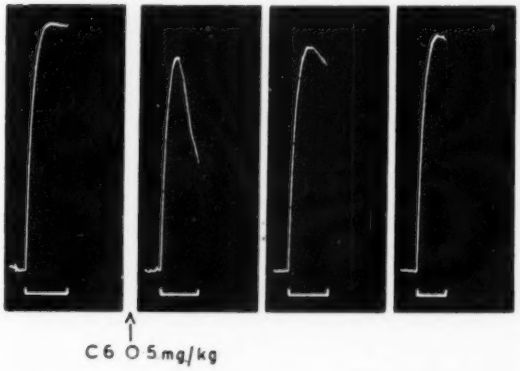

FIG. 2.—Cat, chloralose. Nictitating membrane. Preganglionic excitation at 10/sec. for 30 sec. every 2 min. At arrow, 0.5 mg. hexamethonium iodide per kg. intravenously.

to the drug even during the first 30 sec. of stimulation. Before hexamethonium, the contraction of the membrane was well sustained during the whole of the stimulation period. After the injection of hexamethonium, the contraction height is at first (as we have just seen) little short of the normal; but it soon begins to fall away, and at the end of 30 sec. is reduced to 40 per cent of the normal height. Lastly, we gained the impression that the ganglion became more sensitive if the rate of stimulation was high than with slower frequencies; thus a small dose of pentamethonium (0.02 mg./kg.) was ineffective when the ganglion was excited at 10 shocks/sec. to its preganglionic nerve, but caused a 30 per cent paralysis when excitation at 30/sec. was used.

The duration of action of pentamethonium or hexamethonium on the superior cervical ganglion varied from 3–4 minutes to 20–30 minutes or more according to the dose, and is considerably longer than that of tetraethylammonium; Fig. 1 shows a comparison of the two drugs. Even when the manifest effect of an injection had passed off, the presence of the drug could still be detected 5–15 minutes later by its augmentation of the effects of a similar injection. When precautions were taken to allow for this, it appeared that doubling a given dose of pentamethonium or hexamethonium increased the relaxation of the nictitating membrane by about 20 per cent of the initial contraction height; and that a 20- to 30-fold increase in dose covered the range from a detectable action to a maximal action.

There was never any enhancement by pentamethonium or hexamethonium of the contraction of the nictitating membrane during such an experiment; and, if either drug was injected before excitation of the preganglionic trunk had started, no contraction of the membrane was seen, even with doses as large as 10 mg./kg. This is to be contrasted with the action of such drugs as nicotine or tetramethylammonium, which both augment the contraction of the preganglionically excited nictitating membrane and elicit a contraction when it is not being stimulated.

Site of action

The action of the drug can be shown to be neither on the nictitating membrane nor on the postganglionic trunk. Adrenaline excites as strong a contraction of the membrane after an injection of hexamethonium as before it. In the experiment of Fig. 3, 100 μg. adrenaline were injected intravenously; then the cervical sympathetic was stimulated, and during this excitation, when the contraction of the membrane had reached its peak, 1 mg. hexamethonium iodide was injected, causing complete abolition of the contraction; 100 μg. adrenaline, however, still produced a normal contraction of the membrane.

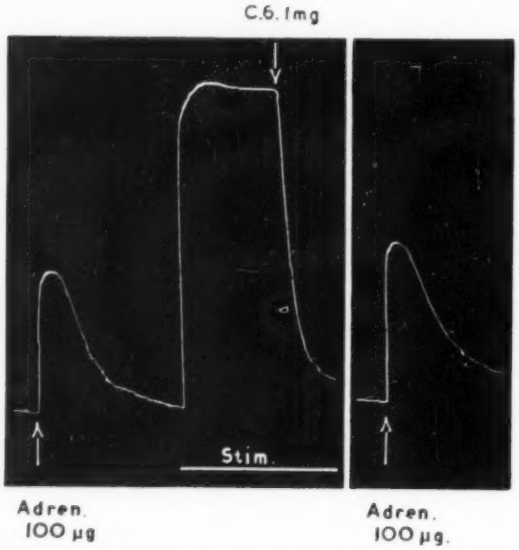

FIG. 3.—Cat, chloralose. 2.9 kg. Nictitating membrane. Contractions elicited by (*a*) 100 μg. adrenaline intravenously; (*b*) preganglionic excitation at 10/sec.; (*c*) 100 μg. adrenaline intravenously. During (*b*), at arrow, 1 mg. hexamethonium iodide intravenously.

A similar experiment was done using stimulation of the postganglionic nerve trunk (Fig. 4). First, postganglionic stimulation was applied. Then the preganglionic trunk was stimulated, and during this excitation 0.75 mg. hexamethonium per kg. was injected; this caused complete relaxation of the nictitating membrane, as shown by

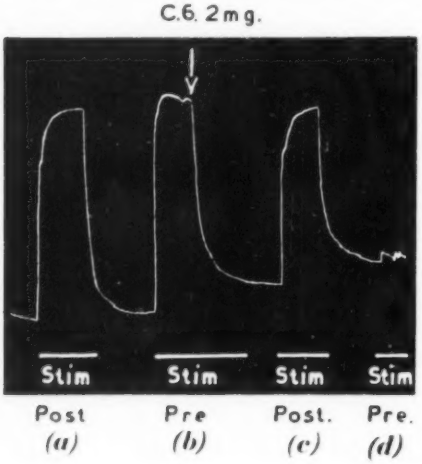

FIG. 4.—Cat, chloralose. 2.7 kg. Nictitating membrane. Contractions elicited by excitation of (*a*) and (*c*) postganglionic trunk, (*b*) and (*d*) preganglionic trunk, at 10/sec. Periods of stimulation shown by continuous line. At arrow in (*b*), 2 mg. hexamethonium iodide intravenously.

the absence of further relaxation when the stimulus ceased. Immediately after this, however, postganglionic stimulation was still able to produce a contraction which reached the same height as before, although a subsequent preganglionic stimulation was ineffectual.

Effect on release of acetylcholine

The superior cervical ganglion was perfused with eserinized Locke's solution and the effluent assayed for acetylcholine on the cat's blood-pressure. Fig. 5 shows the record from this experiment. Before stimulation of the cervical sympathetic, the effluent contained no detectable acetylcholine (certainly less than 3 mμg./c.c.). It was then stimulated for three minutes at a rate of 10/sec., producing a vigorous contraction of the nictitating membrane, and causing the appearance of 11 mμg. acetylcholine, per c.c. in the effluent, with a total output of 49.5 mμg.; inset in the record of nictitating membrane is the depressor effect of 0.5 c.c. of this effluent (Fig. 5*a*). Then 10 mg. pentamethonium iodide was injected into the arterial cannula. Excitation of the nerve was now without effect on the nictitating membrane, but acetylcholine still appeared in the effluent (Fig. 5*b*) in a concentration of 13 mμg./c.c., the total output being 47 mμg., although the dose of pentamethonium was so large that there was sufficient in the effluent to cause a subsequent gradual fall of blood-pressure in the assay cat. Two tests were used to verify that the depressor material was acetylcholine. A sample was made alkaline, stood at room temperature for 20 minutes, neutralized, and reassayed. No depressor activity remained. Another sample was tested after the injection of 0.3 mg. atropine sulphate per kg. into the assay cat. This abolished the depressor effect, without lessening the effect of a small dose of histamine. A dose of pentamethonium, therefore, more than 1,000 times that required to cause some degree of ganglionic block, leaves acetylcholine release in the ganglion entirely unimpaired.

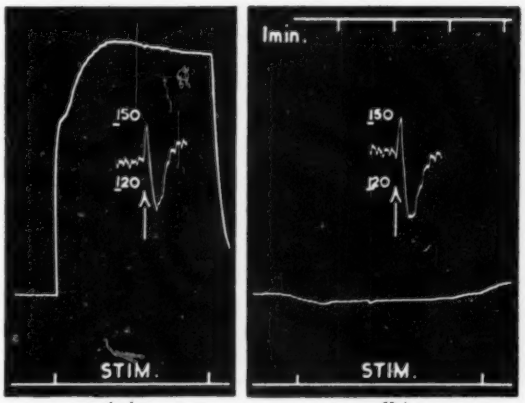

FIG. 5.—Cat, chloralose. Perfused superior cervical ganglion. Contractions of nictitating membrane to preganglionic excitation at 10/sec., and (inset) blood-pressure responses to 0.5 c.c. effluent from ganglion; (*a*) before, (*b*) after the injection of 10 mg. pentamethonium iodide into arterial perfusion cannula.

The same conclusion was reached by doing this experiment slightly differently (Fig. 6). The cervical sympathetic was stimulated continuously and the successive four-minutely volumes of effluent assayed for acetylcholine. During the stimulation, pentamethonium (10 mg.) was injected. A complete paralysis lasting about seven minutes resulted and was followed by a gradual recovery of the contraction of the nictitating membrane. The output of acetylcholine, however, reflected nothing of the paralysis, but fell steadily throughout the experiment, so that it was much less when

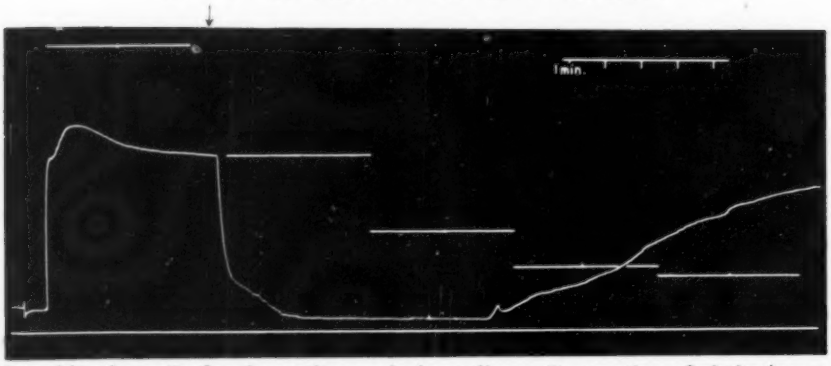

FIG. 6.—Cat, chloralose. Perfused superior cervical ganglion. Contraction of nictitating membrane, and histogram of acetylcholine output from ganglion during continuous excitation at 10/sec. At arrow, 10 mg. hexamethonium iodide into arterial perfusion cannula.

the contraction of the membrane was returning than it had been during the period of complete block. This experiment, therefore, besides showing that the drug does not prevent the release of acetylcholine, also shows that it does not interfere with the processes of acetylcholine synthesis and release which are called into play during sustained preganglionic excitation of the superior cervical ganglion.

Pentamethonium and hexamethonium, therefore, act neither on the organ receiving the postganglionic innervation nor on the nerve trunks or preganglionic nerve terminals, but must interfere, as curare does (Brown and Feldberg, 1936), with the transmission process at the ganglionic synapse itself by raising the threshold for excitation of the ganglion to the acetylcholine released. It is not easy to demonstrate this so clearly for ganglia other than the superior cervical ganglion; but, as will be clear from the experiments still to be described, these compounds have few actions which cannot be explained by this type of ganglionic block, and have many actions which cannot be explained in any other way.

Action on cat's blood-pressure

In a cat anaesthetized with chloralose, pentamethonium or hexamethonium regularly cause a fall of blood-pressure, such as that shown in Fig. 7, when injected. in a dose of 0.2–2 mg./kg. The fall usually starts 5–10 sec. after an intravenous injection, and proceeds (often rather gradually) to reach a maximum in 1–3 min. Just as with the superior cervical ganglia, a comparison with tetraethylammonium shows the effects of the methonium salts to be slower in onset and several times longer in duration; a depressor response due to tetraethylammonium is shown for comparison in Fig. 1.

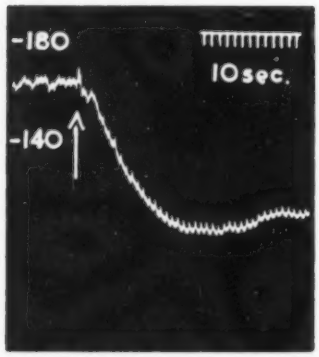

FIG. 7. — Cat, chloralose; blood-pressure. At arrow, 0.3 mg. pentamethonium iodide per kg. intravenously.

The magnitude of this fall depends partly (but not only) on the height of the blood-pressure when the injection is made. Thus, in Fig. 7, when the blood-pressure was 160 mm. at the start, a fall of 60 mm. was obtained; but when the initial blood-pressure was at a lower level, for instance 105 mm. in Fig. 8, the fall due to hexamethonium was only 10 mm.

With still lower pressures, pentamethonium and hexamethonium have even less action, and in the pithed spinal animal they have no depressor action at all. In general, the action of these drugs is to lower the blood-pressure of the chloralosed cat to a certain point, usually about 60–80 mm., but no further, however big the dose (cf., Fig. 8*d*); the effect of increasing the dose beyond that required to produce this maximal effect is simply to prolong the hypotension. With no dose was any pressor effect seen, whether the initial blood-pressure was high or low. Atropine, neoantergan, and vagotomy had no effect on the response.

The depressor action was particularly distinct in animals in which the respiration was depressed. Thus, in one experiment, a dose of decamethonium just sufficient to begin to paralyse the respiratory muscles was given; this led to a typical, slow asphyxial rise in blood-pressure of about 30 mm. mercury during the next two minutes. Hexamethonium (10 mg./kg.), given at this point, caused a big fall of blood-pressure, from 194 mm. to 80 mm., followed by a gradual recovery in the next ten minutes. Similarly, after a dose of thiopentone, which depressed the breathing and caused a similar gradual rise in pressure to 165 mm., pentamethonium (10 mg./kg.) caused an abrupt fall in pressure to 40 mm., with recovery during the next 10–15 minutes. Later in the same experiment, the same dose of thiopentone was given, but with simultaneous artificial respiration; pentamethonium was then relatively slow in its action, and lowered the blood-pressure only to 80 mm.

This intimate relationship between the action of pentamethonium or hexamethonium and the height of the blood-pressure at the moment when one of these drugs is injected, together with their complete ineffectiveness when the blood-pressure is below a certain level, point directly to the proba-

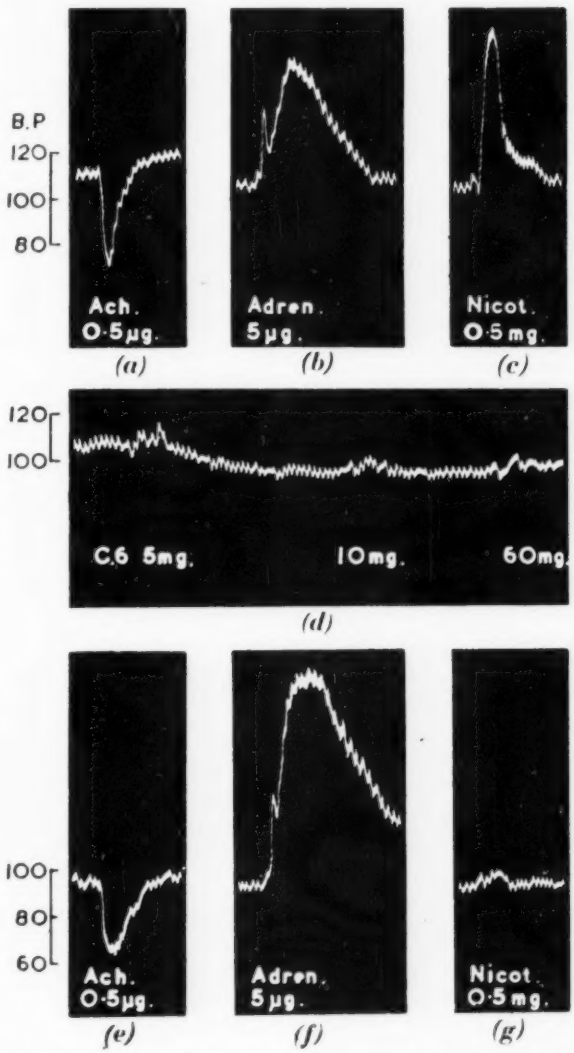

FIG. 8.—Cat, chloralose; blood-pressure. Intravenous injections: (*a*) and (*e*) acetylcholine, 0.5 µg.; (*b*) and (*f*) adrenaline, 5 µg.; (*c*) and (*g*) nicotine, 0.5 mg.; (*d*) hexamethonium iodide, 5 mg., 10 mg., 60 mg. in succession.

bility that they produce their depressor effect by releasing sympathetic vascular tone. This was confirmed by finding that they prevent the action of substances such as nicotine or tetramethylammonium which, by exciting the autonomic ganglia, increase the intensity of the vasoconstrictor discharge from ganglia controlling the blood-pressure. Fig. 8 shows the effect of 75 mg. hexamethonium in abolishing the effects of nicotine. Fig. 9 shows a smaller dose abolishing the action of a similar dose of nicotine, and greatly attenuating the vigorous pressor response to a big dose of tetramethylammonium iodide. On the other hand, in Fig. 8 it can also be seen, for instance, that a large dose of hexamethonium had but little effect on the action of 0.5 μg. acetylcholine. Such effect as is visible can be attributed simply to the lowering of the general blood-pressure; and the level to which the acetylcholine reduced the blood-pressure was even lower after hexamethonium than before. The duration of the acetylcholine action was actually somewhat prolonged. Similarly, the effect of 5 μg. adrenaline was not reduced. On the contrary, the pressor action of this dose was nearly twice as great as before the injection of hexamethonium, and its action was correspondingly longer lasting.

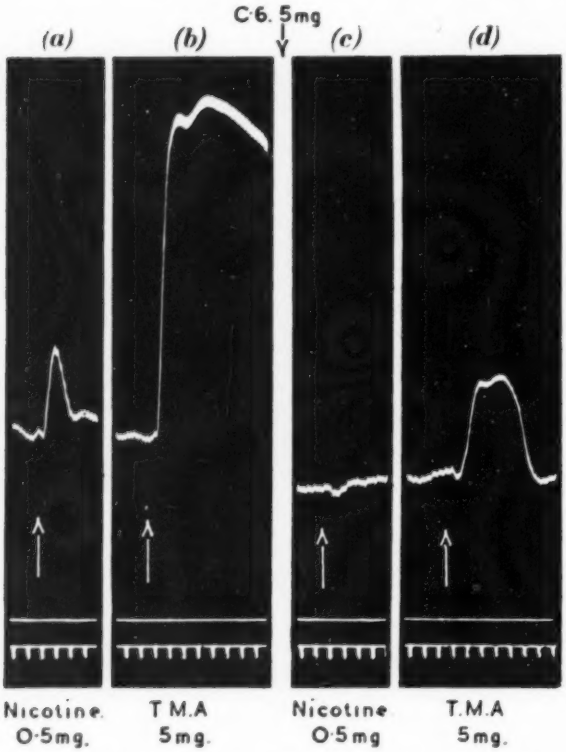

FIG. 9.—Cat, chloralose, 2.5 kg.; blood-pressure. Intravenous injections: (*a*) and (*c*) nicotine, 0.5 mg.; (*b*) and (*d*) tetramethylammonium iodide, 5 mg. Between (*b*) and (*c*) hexamethonium iodide, 5 mg.

These experiments on the cat's blood-pressure provide, therefore, further evidence for our belief that pentamethonium and hexamethonium act solely by paralysing autonomic ganglia, and that they are devoid of action on the effector cells supplied by the efferent fibres from such ganglia. Since, in the absence of autonomic tone, these drugs cause no depressor response, either prompt or delayed, and since they do not depress the vascular responses to acetylcholine and adrenaline, it can now also be said that they are free from muscarine-like, histamine-liberating, atropine-like, or adrenolytic actions, although all these properties are known to exist in other compounds of quaternary nitrogen.

Observations incidental to our main experiments have exemplified how ganglionic block by pentamethonium or hexamethonium may depress reflex cardiovascular responses. For instance, when an animal is asphyxiated, reflex mechanisms normally bring about a considerable rise in blood-pressure (60–100 mm.), with pronounced cardioacceleration; but after an injection of pentamethonium or hexamethonium

this response is reduced and may even be absent. On the other hand, these reflex mechanisms also serve to limit such changes in blood-pressure in the normal animal as those caused by the injection of acetylcholine or adrenaline; hence the injection of hexamethonium actually increases and prolongs the action of adrenaline and acetylcholine.

Action on effects of vagal stimulation

Pentamethonium and hexamethonium will also inhibit the effects of stimulation of the peripheral stump of the vagus, cut in the neck, on the heart rate and blood-pressure. In the experiment of Fig. 10, the right vagus was faradized for 10 sec.,

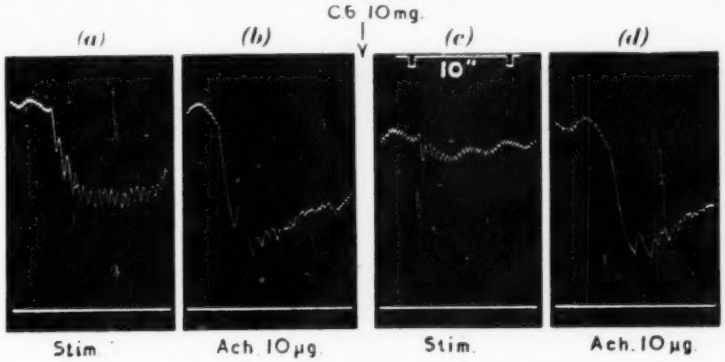

FIG. 10.—Cat, chloralose; blood-pressure. Intravenous injections: (*a*) and (*c*) right vagus stimulated at 10/sec. for 10 sec.; (*b*) and (*d*) injection of 10 µg. acetylcholine. Between (*b*) and (*c*) 10 mg. hexamethonium iodide.

causing a fall in blood-pressure and considerable slowing of the heart; 10 µg. acetylcholine caused a similar response. Then 10 mg. pentamethonium was injected intravenously. Two minutes later, faradization of the vagus was ineffective, although acetylcholine had the same action as previously. Exactly the same results were obtained when hexamethonium or tetramethonium was used. These experiments again provide evidence that the action of these drugs is restricted to ganglia, and does not extend to the effector organ.

Effect on the heat output of the rabbit's ear

The heat-flow meter, recently devised by Hatfield (1950), offered a simple means of recording an increase in blood flow through a rabbit's ear as an increase in the rate of loss of heat from the ear to the environment. Fig. 11 shows the result of injection of 10 mg. pentamethonium iodide per kg. into one ear vein of an animal to whose other ear the recording disk was fastened. After a brief latency, the heat output of the ear rose abruptly,

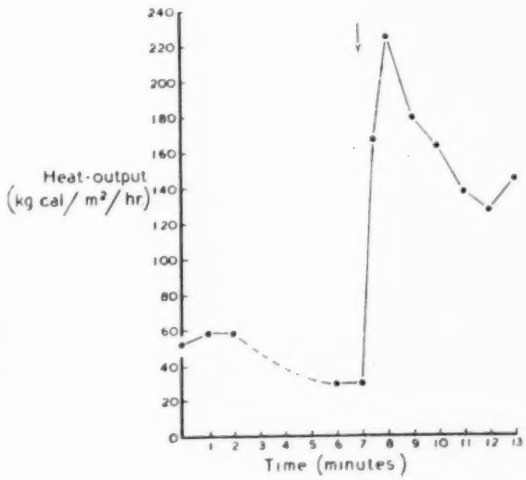

FIG. 11.—Rabbit. Heat output from the ear, recorded by Hatfield heat-flow meter. At arrow, 10 mg. pentamethonium iodide per kg. intravenously.

and then more slowly fell again, synchronously with the visible flushing of the ear. If the ear was already flushed, because, for instance, of the warmth of the surroundings, this rise in heat output was much smaller, and it became more prominent as the ear was colder and more vasoconstricted. Just as with the cat's blood-pressure, the action of pentamethonium in increasing the blood-flow in the rabbit's ear requires the presence initially of some degree of sympathetic tone.

Effects on small intestine

Rabbit ileum.—As we have previously described, pentamethonium and hexamethonium are very active in paralysing the peristaltic reflex of the rabbit's or guinea-pig's isolated ileum, which can be elicited by raising the pressure within the lumen of the intestine. Experiments of this kind showed at the same time how these drugs neither themselves caused any longitudinal contraction nor altered the myogenic, longitudinal contraction in response to the rise in pressure. Even with concentrations as high as 1 mg./c.c. we obtained no action on the longitudinal contraction of the ileum. The effect on the peristaltic reflex, however, was obtained with small doses; and the effect tended to persist after washing the drug out. Thus, a dose of 0.1 mg. pentamethonium in a bath of volume 100 c.c. produced a 50 per cent reduction of the peristaltic contractions. The reflex was still depressed 10 min. after the drug had been washed out. With tetraethylammonium, on the other hand, a dose of 0.7 mg. was required to produce the same effect, and the reflex was rapidly restored to normal after a single washout.

Cat ileum.—An attempt was made to demonstrate the same phenomenon in the ileum of the chloralosed cat, leaving the intestine *in situ*, so that the effect of a drug on the nictitating membrane and on the peristaltic reflex could be compared simultaneously. But effects of a different kind are obtained in this preparation (Fig. 12).

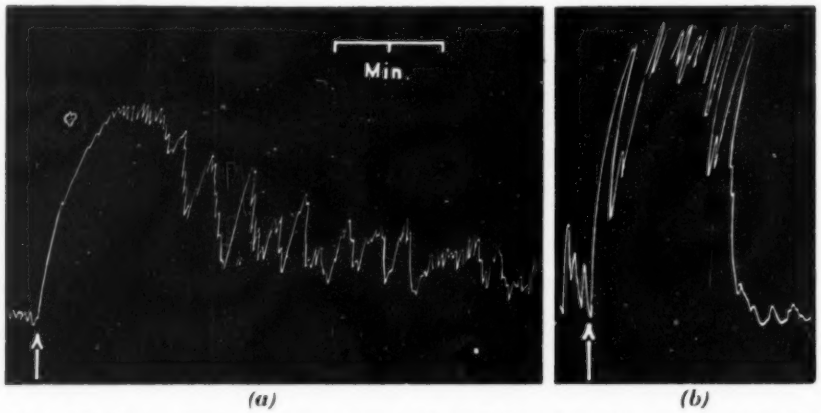

FIG. 12.—Cat, chloralose. Record of movements of small intestine. Intravenous injections. (*a*) At arrow, 0.24 mg. hexamethonium iodide per kg. (*b*) At arrow, 1.5 tetraethylammonium iodide per kg.

After the injection of 0.24 mg. hexamethonium, the rhythmic activity of the intestine is increased greatly in amplitude. During this period the gut was the site of very vigorous, circular contractions, without the progressive quality of the peristaltic

wave. Precisely the same effects (also shown in Fig. 12) were obtained with tetraethylammonium (1.5 mg.). It was impossible to say, from these experiments, what, in fact, had happened to the peristaltic reflex. Bayliss and Starling (1900) showed that, not only was the sympathetic innervation of the cat intestine inhibitory in function, but that vagal excitation might also have an inhibitory action; and that local enteric reflexes easy to display in the rabbit were, in the cat, difficult to demonstrate. There is no reason, therefore, to suppose that the effects we have observed in the cat are due to anything other than the release of a strong sympathetic and possibly vagal tone inhibitory to a predominantly myogenic, rhythmic activity of the intestine.

Other actions

All the actions we have so far described can be attributed to these drugs blocking the action of acetylcholine released at the ganglionic synapse by a preganglionic nerve volley. This is, in fact, far the most important action which they display; in addition, a similar, but much feebler, antagonism to acetylcholine, and related drugs, can be shown at the neuromuscular junction and on the frog's rectus muscle. This antagonism has already been described in our earlier paper. Occasionally, and only with very large doses, a slowing of respiration of the chloralosed cat was observed. An example may be found in Fig. 8, in which the rhythmic fluctuation of the blood-pressure due to the respiration can be seen to become slightly less frequent for a minute or two after the last large dose of hexamethonium. This slowing of respiration may be associated with some transient reduction of the respiratory minute volume. We have not analysed this effect, nor have we excluded interference with pulmonary reflexes as the cause of it.

Our previous paper reported our results on the toxicity of these compounds, and it should be referred to for details. In mice and rats pentamethonium and hexamethonium had an LD50 of 50–90 mg./kg. when the drugs were injected intravenously. Rabbits tolerated doses of more than 40 mg./kg. intravenously. Further, rabbits receiving 10 mg. hexamethonium per kg. intravenously every day for a month did not lose weight or suffer in health, and autopsy showed no abnormalities attributable to the drug.

Discussion

All the properties of these members of the methonium series can be ascribed to their power of raising the threshold of the ganglion cell, and in far less degree of the motor endplate, to acetylcholine or to drugs acting like acetylcholine. They can block the superior cervical ganglia, the ganglia maintaining sympathetic vascular tone, those mediating the effects of the vagus on the heart, and the peristaltic reflex of the intestine. Recent work (Paton and Perry, 1950) has shown that they do not depolarize the ganglion cell, as do nicotine and tetramethylammonium, but are pure blocking agents of the same kind as *d*-tubocurarine or tetraethylammonium. On the other hand, they have no direct action on smooth muscle, such as that of the intestine, nictitating membrane or blood-vessels, nor on the heart; they do not interfere with the vascular actions of adrenaline or acetylcholine; they do not affect striated muscle directly; they do not excite autonomic ganglia; they do not interfere with the release

of acetylcholine, nor with the processes of synthesis involved in prolonged stimulation of the superior ganglia.

The high potency of these compounds, and their remarkable specificity, suggests that they may provide a useful physiological tool. The absence of neuromuscular activity and of histamine liberation in particular, makes their use more convenient than that of *d*-tubocurarine, which is in other respects an efficient ganglion-blocking agent. They lack, too, the side-actions of tetraethylammonium ion, such as the intense parasthesiae which result from an intravenous injection, and its occasional pressor effect, said to be due to the release of an adrenaline-like substance (Page, 1949). Their specificity has, indeed, already been of use in the analysis of the action of compound 2688F (Ambache, 1949) and of the action of drugs stimulating the small intestine (Feldberg, 1950).

Relative potency in different tests

In our previous paper, quantitative data were given for the activity of these compounds on two test objects, the superior cervical ganglion and the peristaltic reflex of the rabbit ileum. These figures are shown again in Table I. Inspection of the potencies estimated by the two tests reveals the interesting fact that the ratio of the

TABLE I

RELATIVE POTENCY OF METHONIUM SALTS ON SUPERIOR CERVICAL GANGLION AND ON PERISTALTIC REFLEX

Relative potency (C6 = 100)	C3	C4	C5	C6	C7	T.E.A.
(*a*) Cn superior cervical ganglion	<1.0	2.0	80.0	100.0	10.0	5.0
(*b*) Cn peristaltic reflex of ileum	4.3	5.9	33.3	100.0	16.7	14.0
(*a*) ÷ (*b*)	<0.2	0.34	2.4	1.0	0.6	0.36

potencies in the two tests varies considerably from one compound to another. The extremes are pentamethonium and C3; for the ratio is 2.4 for pentamethonium, but is less than 0.2 for C3; i.e., pentamethonium is proportionally more effective on the sympathetic ganglion and C3 on the peristaltic reflex. Taking all the compounds tested, they can be arranged (in order of increasing action on the ileum relative to the superior cervical ganglion): pentamethonium—hexamethonium—tetraethylammonium—tetramethonium—C3.

The comparison just quoted is between ganglia of considerably different structure and function. But even between more closely related ganglia, such as the superior cervical ganglion and those maintaining vasomotor tone, significant differences appear; Fig. 1 shows how a dose of hexamethonium may have a considerable effect on the superior cervical ganglion and none on the blood-pressure, although a dose of tetraethylammonium with a less sustained effect on the cervical ganglion caused a substantial fall in blood-pressure. The extent of these variations in sensitivity remains to be discovered. It presents, already, an interesting parallel to the discrepancies in blocking actions at the ganglionic and neuromuscular synapses recently recorded by many workers. There may be latent in such differences the possibility of obtaining, with different ganglionic blocking agents, an action directed specifically at a particular set of ganglia within the autonomic system.

Relation of activity to chemical structure

It is still premature to attempt to relate the chemical structure of known ganglion-blocking drugs to their pharmacological activity. For instance, among salts containing methylated quaternary nitrogen are several highly effective compounds (e.g., pentamethonium, hexamethonium, *d*-tubocurarine); but ganglion-blocking activity occurs also among ethylated quaternary salts (e.g., tetraethylammonium, bistriethylammonium decane, 2512F). Again, although a five or six carbon atom methylene chain produces the highest potency in the bistrimethylammonium series, it causes almost the lowest activity in the bistriethylammonium series (Chou and de Elio, 1947). It seems likely that order will not be achieved among such flagrant inconsistencies until there has been further analysis of the details of the action of the various compounds concerned.

In a discussion (Paton and Zaimis, 1949a) of the fact that decamethonium is the most active member of the methonium series in producing neuromuscular block, it was argued that the evidence indicated that the drug acted by forming a link at each end of its molecule with receptor groups spaced the corresponding distance apart. On such a theory, the ten carbon atom chain is a measure of the inter-receptor distance. Similar arguments may be invoked here, for, as with decamethonium, there appears to be no other physical property of the molecule competent to change its activity by a factor of 40 when a single carbon atom is removed from the polymethylene chain. We suggest, then, that we are again measuring an inter-receptor distance, but it is quite different from that at the motor endplate. Despite the profound analogies between transmission processes at the myoneural and at the ganglionic synapses, there are evidently significant and fundamental differences between them.

SUMMARY

1. The shorter chain members of the polymethylene bistrimethylammonium series cause ganglionic block. The pentane and hexane derivatives, pentamethonium and hexamethonium, are particularly effective.

2. Pentamethonium and hexamethonium do not affect the response of the nictitating membrane to adrenaline or to stimulation of the postganglionic trunk of the superior cervical ganglion. Injected into the perfused ganglion, they do not affect the release of acetylcholine produced by stimulating the preganglionic trunk. They do not cause any initial excitation of the ganglion. They paralyse ganglionic transmission solely by raising the threshold of the ganglion cell to the acetylcholine released at preganglionic nerve endings.

3. Intravenous injections of pentamethonium or hexamethonium cause a fall of cat's blood-pressure which is greater the higher the initial level of pressure; they also cause vasodilatation of the rabbit's ear. They abolish or reduce the pressor action of nicotine and of tetramethylammonium, and reduce the pressor response to asphyxia. The action of acetylcholine on the blood-pressure is not reduced by large doses of hexamethonium, but is slightly prolonged; that of adrenaline is considerably enhanced and prolonged. The vascular actions of pentamethonium and hexamethonium are entirely attributable to removal by these drugs of autonomic vascular control.

4. Slowing of the heart and fall in blood-pressure produced by peripheral vagal stimulation are abolished by pentamethonium and by hexamethonium, without any reduction of the effects of an initially equiactive dose of acetylcholine.

5. Pentamethonium and hexamethonium abolish the peristaltic reflex of the isolated rabbit intestine. When they are injected intravenously into a cat anaesthetized with chloralose, they arouse vigorous localized activity of the circular muscle in the small intestine; tetraethylammonium shares this action.

6. The relative potency of the methonium salts in paralysing ganglionic transmission varies according to the nature and activity of the ganglion studied.

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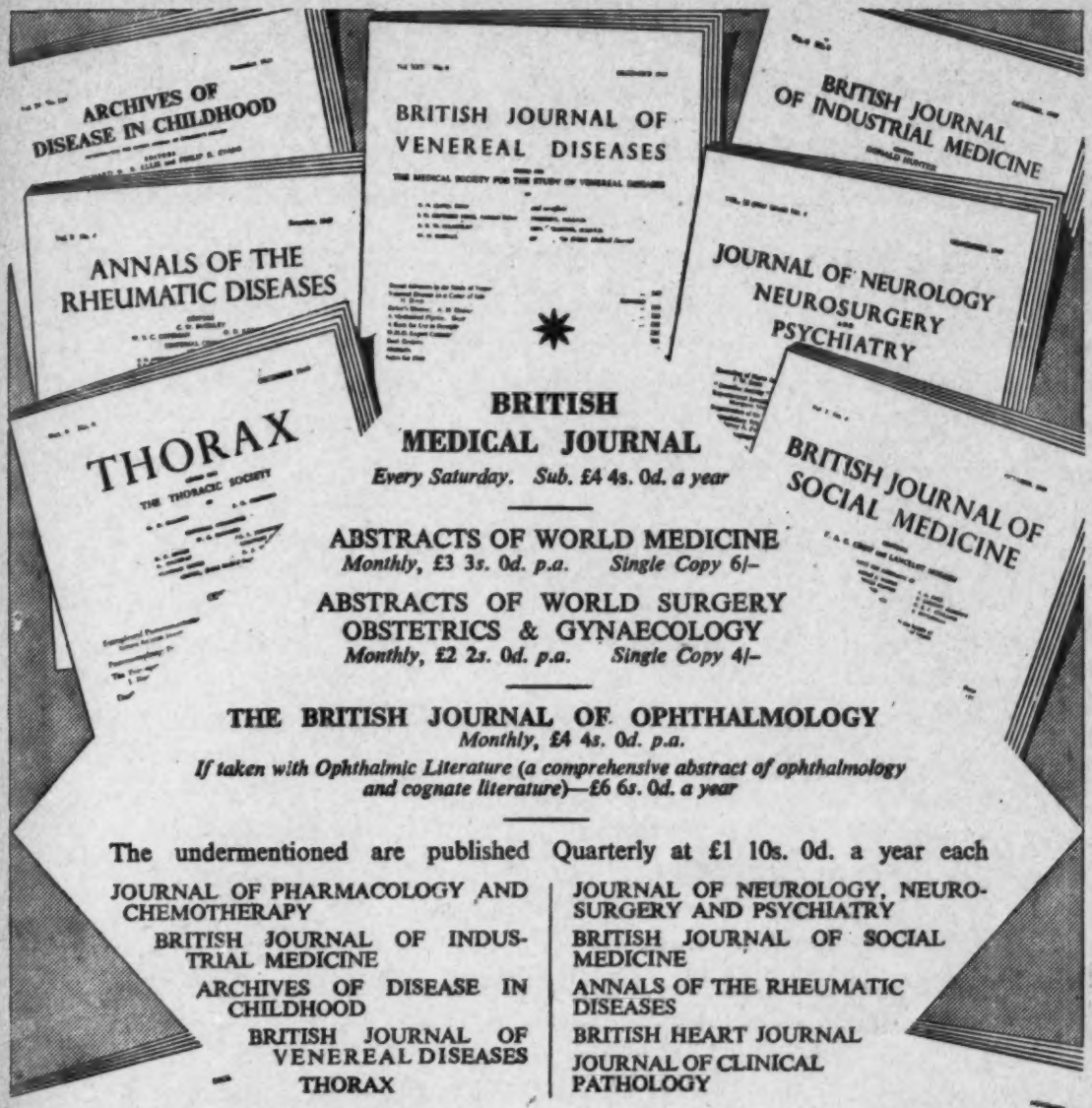
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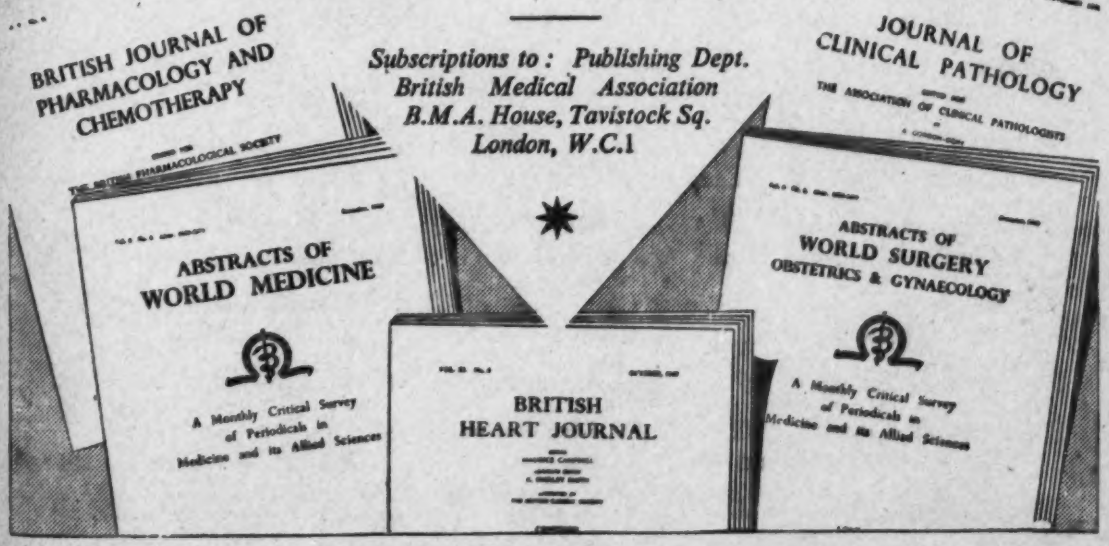
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CONTENTS

PAGE

Central Nervous Function and Changes in Brain Metabolite Concentration: Characteristic Glycogen Increment Patterns Produced by Convulsant Drugs M. R. A. CHANCE 1

The Quantitative Separation of Adrenaline and *Nor*adrenaline in Biological Fluids and Tissue Extracts
T. B. B. CRAWFORD and A. S. OUTSCHOORN 8

Inhibition of Cholinesterases by Irreversible Inhibitors *in vitro* and *in vivo*
F. HOBBIGER 21

Susceptibility to Ventricular Fibrillation during Chloroform and Cyclopropane Anaesthesia D. E. HUTCHEON 31

Estimation and Photolability of Some Thiosemicarbazones A. SPINKS 35

Unmasking, after Cholinergic Paralysis by Botulinum Toxin, of a Reversed Action of Nicotine on the Mammalian Intestine, Revealing the Probable Presence of Local Inhibitory Ganglion Cells in the Enteric Plexuses
N. AMBACHE 51

Analysis of Certain Interactions of Nicotine with Bradykinin and Histamine
N. AMBACHE and M. ROCHA E SILVA 68

Responses of Coronary Vessels to Pituitary (Posterior Lobe) Extract and to Adrenaline PAMELA KORDIK 75

The Nature of Uterine and Intestinal Sympathin
MONICA MANN and G. B. WEST 79

The Activity of Thiosemicarbazones Alone and in Combination with Other Drugs in Experimental Corneal Tuberculosis
R. J. W. REES and J. M. ROBSON 83

The Enhancement of the Effect of Oral Penicillin in Laboratory Animals by the Use of Oil Containing Aluminium Resinate
Y. C. HSU and G. A. H. BUTTLE 89

*Nor*adrenaline and Adrenaline in Vessels of the Rabbit Ear in Relation to the Action of Amine Oxidase J. H. BURN and JUDITH ROBINSON 101

Reversal of the Vascular Response to Acetylcholine and Adrenaline
J. H. BURN and JUDITH ROBINSON 110

Biological Estimation of Adrenaline and *Nor*adrenaline in Tissue Extracts Contaminated with Histamine or Acetylcholine CARL G. SCHMITERLÖW 120

The Formation *in vivo* of *Nor*adrenaline from 3: 4-Dihydroxyphenylserine (*Nor*adrenaline Carboxylic Acid) CARL G. SCHMITERLÖW 127

The Walker Carcinoma 256 in the Screening of Tumour Inhibitors
A. L. WALPOLE 135

Pharmacological Study of Injected Cholinesterase I. T. BECK 144

Paralysis of Autonomic Ganglia by Methonium Salts
W. D. M. PATON and E. J. ZAIMIS 155

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